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SANITARY SIGNIFICANCE OF FECAL COLIFORMS IN THE ENVIRONMENT

E. E. Geldreich

Robert A. Taft Sanitary Engineering Center
Cincinnati, Ohio

November 1966

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SANITARY SIGNIFICANCE
OF
FECAL COLIFORMS
IN THE ENVIRONMENT

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U. S. DEPARTMENT OF THE INTERIOR
Federal Water Pollution Control Administration

November 1966

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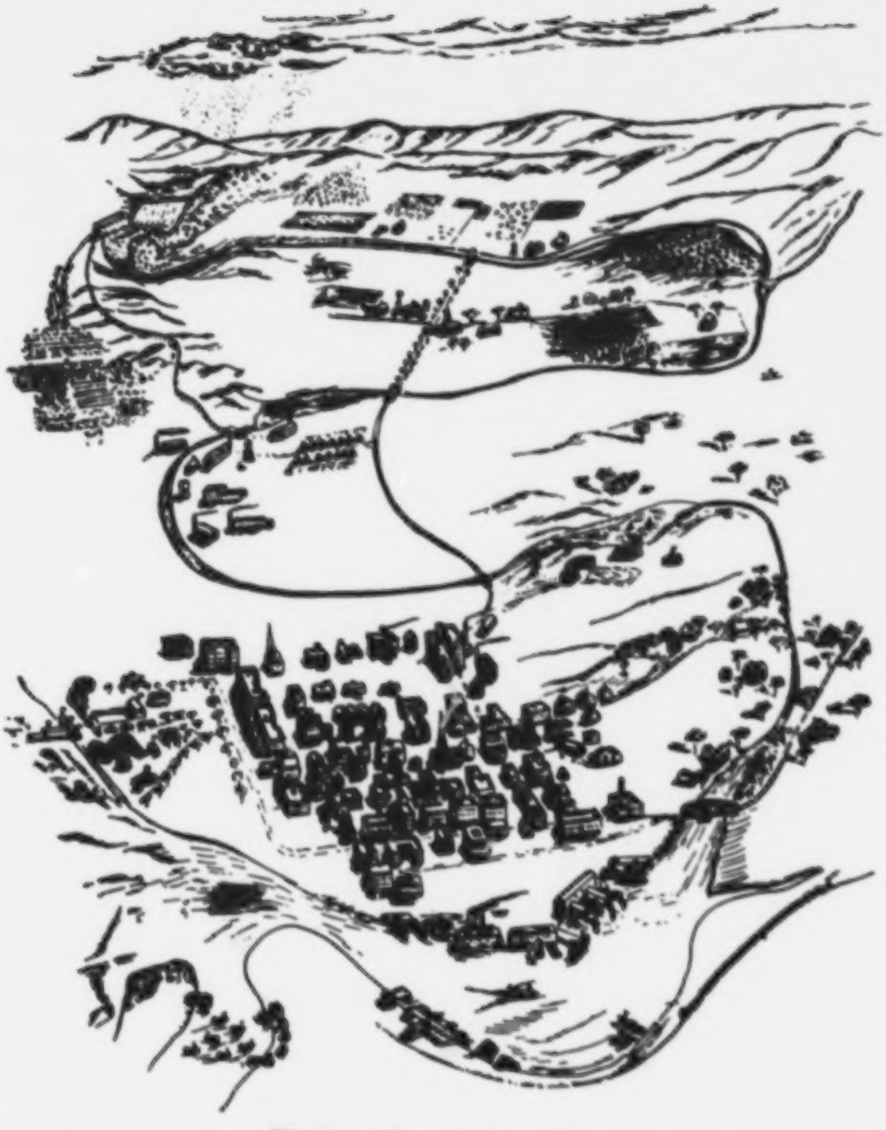

The Life History of a Stream

Reprinted: Courtesy of

Phelps, E. B., *Stream Sanitation*, John Wiley and Sons, Inc., New York, 1944.

FOREWORD

The science of sanitary bacteriology was born when Escherich in 1885 discovered bacteria characteristic of human feces and showed that such microorganisms in water indicated fecal contamination. In the years following, much information was accumulated on the physiology of coliform bacteria, their natural occurrence, survival in water, and sanitary significance. Although several investigators threw some light upon the difficult question of the sanitary significance of fecal coliforms in the environment, no definitive interpretation appeared. Studies in recent years have only emphasized the complexity of this enteric group.

This report is important because it emphasizes the value of the fecal-coliform detection procedure in investigations of river pollution, sewage-treatment systems, and bathing-water quality. The report gathers together in one volume the results of past and present research conducted by the Microbiological Activities of the Basic and Applied Sciences Program at the Cincinnati Water Research Laboratory (Robert A. Taft Sanitary Engineering Center), Cincinnati, Ohio, on the significance of fecal coliforms

Inasmuch as the fecal-coliform detection procedure has received much attention in legal hearings and enforcement conferences on pollution control, it seemed desirable to review the current status of the test. With these aims in mind, the research publications are presented as an informative reference for the increasing numbers of bacteriologists and sanitary engineers involved in stream-pollution studies.

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ACKNOWLEDGMENTS

It is to the credit of E. E. Geldreich's and H. F. Clark's (deceased) initiative and clear recognition of the importance of this subject that this volume exists. Our thanks are due to G. M. Gigliotti for his efforts in compiling these papers. The publications are the collaborative efforts of several colleagues, L. C. Best, R. H. Bordner, C. B. Huff, and B. A. Kenner, who have co-authored these contributions. Especially helpful was the stimulating support of Dr. N. A. Clarke, Chief, Microbiological Activities, Basic and Applied Sciences Program, Federal Water Pollution Control Administration.

The combined efforts of personnel in various water-treatment plants throughout the country and the technicians who gave bacteriological assistance are appreciated.

We are indebted to the editors of the *Applied Microbiology*, *Journal American Water Works Association*, *Journal Applied Bacteriology*, *Journal of Water Pollution Control Federation*, *Public Health Reports*, and *Principles and Applications in Aquatic Microbiology* in which many of these papers were previously published.

CONTENTS

Page
- **Abstract** ... vii
- **Introduction** ... 1
- **Chapter I. Background** ... 3
  - The Gas-Ratio (Hydrogen-to-Carbon Dioxide) Differential Test ... 4
  - The Methyl Red Test ... 5
  - The Indole Reaction ... 6
    - Recommended Indole Test Procedure ... 7
    - Discussion ... 7
    - Interpretation and Use ... 8
    - Summation ... 8
  - The Voges-Proskauer (Acetylmethylcarbinol) Test ... 8
    - Recommended Test Procedure ... 9
    - Interpretation and Use ... 9
    - Stability of the Voges-Proskauer Test ... 10
    - Summation ... 10
  - The Citrate Test ... 10
    - Recommended Test Procedure ... 12
    - Interpretation and Use ... 12
    - Summation ... 12
  - The IMViC Classification ... 12
  - Conditions For Acceptance of IMViC Classification ... 13
    - Discussion ... 14
    - Summation ... 14
  - The Elevated Temperature Test ... 14
  - Separation of Fecal From the Non-fecal Coliform Groups ... 17
  - Definitions of Methodology ... 18
  - Discussion of Tests ... 20
  - Summation ... 21
- **Chapter II. Fecal-Coliform Group — Techniques** ... 23
  - Multiple Tube Procedure ... 23
    - Boric Acid Lactose Broth ... 23
      - Materials and Methods ... 24
      - Results ... 25
      - Discussion ... 26
      - Summation ... 30
    - EC Medium ... 30
      - Materials and Methods ... 31
      - Results ... 31
      - Discussion ... 31
      - Summation ... 33

Membrane Filter Procedures 33
  Improved Techniques 33
  Procedure 34
  Evaluation 35
  Results 35
  Discussion 39
Evaluation of Methods 41
  Origins of Coliform Organisms 42
  Suggested Criteria 42
  Standard Tests 43
  Source Differentiation 43
  Test Precision 44
  Superior Test Procedure 44
  Summation 45
Chapter III. Warm-Blooded Animals 47
Methods 47
Results 48
Discussion 51
Summation 54
Chapter IV. Fresh-Water Fish 57
Material and Methods 58
Results and Discussion 59
Chapter V. Vegetation and Insects 73
Materials and Methods 74
Results and Discussion 75
Chapter VI. Soils 85
Materials and Methods 86
  Sources and Types of Samples 86
  Sampling Procdure and Examination of Sampling 86
Results 87
Discussion 89
Chapter VII. Water 93
Drinking Water 93
  Sanitary Significance 94
  Summation 94
Sewage and Stabilization Ponds 95
  Methods 96
  Results 98
  Discussion 101
  Summation 103
Storm Water 104
Review 105
References 111
Index 119

ABSTRACT

This compilation of previously published research papers by personnel of the Microbiological Activities, Basic and Applied Sciences Program, Cincinnati Water Research Laboratory, (Robert A. Taft Sanitary Engineering Center), brings together in one volume much of the information concerning fecal-coliform bacteria that has appeared in various scientific journals. The material is organized into seven chapters that include background; methodology; and occurrences in warm-blooded animals including man; fresh-water fish; vegetation and insects; and soil. The final section covers the interpretation and significance of fecal-coliform bacteria in water pollution studies.

INTRODUCTION

As a river moves from its inception in snow melt, in the outpouring of underground springs, or in rainfall runoff, it comes in contact with many environments. As it falls to the earth's surface, rain or snow picks up particulate material with attached bacteria. Soil itself contributes a wide variety of bacteria to storm-water drainage, the number and species being related to contamination of the drainage area and to the quantity and frequency of precipitation. In addition to bacterial contamination from soil, chemical compounds from mineral sources and mine drainage drastically alter the chemical purity of water. These changes then directly or indirectly influence the bacteriological flora. Finally, man and his culture tremendously change the stream by adding domestic sewage and industrial wastes. These are the various environments that add contaminants to the water menstruum. In so doing they become a part of the water itself and thereby influence the survival of bacterial organisms, some of which are pathogenic to man.

Most bacteria in water are derived from contact with air, soil, living and decaying plants or animals, and the fecal excrement from warm-blooded and cold-blooded animals. These bacteria have been shown to be aerobic and anaerobic spore-forming bacilli associated with varying densities of coliforms, fecal coliforms, fecal streptococci, staphylococci, chromogenic forms, fluorescent strains, nitrifying and denitrifying groups, iron and sulfur bacteria, and proteus species, plus an unpredictable occurrence of pathogenic bacteria. Although the list of bacterial species found in water could easily be expanded to include many others, such a compendium would be of little value. Many bacteria are without sanitary significance since they die rapidly in water, come from various unknown sources, are widely distributed in the natural environment, or have no known or suspected association with human or animal wastes. Therefore, major emphasis has been on studies of those species or groups of bacteria present in water that are related to sources of pollution considered a hazard to public health.

The access of fecal pollution to water may add a variety of intestinal pathogens at any time, and at one time or another, enteric pathogenic bacteria probably will be present. Most common genera of pathogenic organisms found in water are: *Salmonella*, *Shigella*, *Vibrio*, *Mycobacterium*, *Pasteurella* and *Leptospira*. Although these pathogens have been found, a wide variety of media and methods is necessary to detect them. Recent qualitative studies on *Salmonella* detection in sewage, irrigation water, river water, and tidal waters produced promising techniques that permit the isolation of these pathogens from waters with relatively low coliform densities. Quantitative reproducibility of methods for pathogenic bacteria is still in the developmental stage, however. The use of these organisms as pollution indicators is not desirable because the intervals between sampling, pathogenic detection, and condemning a water supply result in certain exposure risks to the consumer, and because failure to demonstrate pathogenic organisms does not ensure a safe water.

Water contaminated by fecal pollution is subject to the probability of enteric viruses excreted from these same warm-blooded animals. With improved techniques developed in recent years, various enteric viruses have been isolated from the polluted-water environment. The densities of viruses present in human feces and sewage are of a much lower magnitude, however, than those for coliform bacteria.

Currently, various studies of the origins of fecal-coliform bacteria have generated renewed interest in this group as a better indicator of pollution by warm-blooded animals than the traditional total coliform procedure when applied to stream pollution investigations, sewage treatment systems, and bathing water quality. This volume is presented as a compilation of data on fecal-coliform bacteria, their definition, methodology, occurrence in the environment, and sanitary significance in water pollution.

CHAPTER I. BACKGROUND*

Use of the presence of bacteria as an indicator of the sanitary quality of water originated in 1880 with the description by von Fritsch of *Klebsiella pneumoniae* and *K. rhinoscleromatis* as microorganisms characteristic of human contamination. A short time later Escherich added his *Bacillus coli* as an indicator of fecal pollution. A careful study of their writings shows that they considered human feces as dangerous pollution, while the feces of other warm-blooded animals were not a hazard to mankind. Apparently this was the obscure origin of the science of sanitary water bacteriology, and from this source the current "coliform group" has developed with its methods for measurement, its various classifications, procedures, and complex, but, at times, somewhat controversial systems of interpretation.

Escherich(*1*), by the use of fermentation reactions in glucose and in milk media, morphology, and motility, divided his *B. coli* into two species, which he named *B. coli-commune* and *B. lactis-aerogenes*. Wurtz(*2*) recommended the addition of indicators to the culture medium to demonstrate acid production by the coliform group in the fermentation of carbohydrates. Smith(*3*) established the theoretical basis for the bacteriological examination of water and, on the basis of fermentation of sucrose, divided *B. coli-commune* (Escherich) into two species, which he named the alpha and beta types of *B. coli-communis*. A significant contribution was made by Dunham(*4*) when he devised the inverted vial in liquid medium for measurement of gas produced by bacterial fermentation of carbohydrates. A period of approximately 15 years followed in which investigators applied all available physical and biochemical procedures, with special emphasis on carbohydrate reactions; this resulted in detailed classifications of the coliform group. Space limitations permit citing the work of only a few investigators during this time. Ford(*5*) recommended fermentation reactions in glucose, lactose, and sucrose; MacConkey(*6, 7*) described methods for division of the coliform group into 128 possible types and actually isolated 36 types; Bergey and Deehan(*8*) expanded the classification to include 16 groups composed of 256 types; and Jackson(*9*), displeased with all the fine subdivision of the coliform group, proposed retaining the 4 original basic groups of MacConkey(*6*) by using sucrose and dulcitol fermentation reactions and adding the fermentation in raffinose and mannitol to give a total of 16 groups. The Jackson classification was adopted by the Committee on Standard Methods in 1912. Kligler(*10*) expressed dissatisfaction with the 1912 *Standard Methods* recommendation and further reduced the number of groups from 16 to 4 by recommending only sucrose and salicin reactions.

By this time much information had been accumulated on the biochemical and physical characteristics of the coliform group. From the viewpoint of the taxonomist, it was a well-classified group with numerous species and strain divisions. From a sanitary consideration, however, the correlation between the sources of coliform bacteria such as fecal, soils, plants, etc., and their tabulated characteristics left much to be desired for public-health interpretation. Eijkman(*11*), in dissenting with the current trend, claimed that such classifications were not serving the public interest in water-quality control; he believed the major objective should be measurement of fecal content in food and water rather than estimation of all the coliform bacteria present. He divided the coliform group into a fecal group that would produce gas from glucose at 46°C and a non-fecal group that would produce no change in the medium under the conditions described for the test. He further defined his fecal-coliform group, which was positive by the Eijkman test, as the coliform bacteria from the gut of warm-blooded animals, and the non-fecal group as all coliforms derived from other sources. His test procedure was highly specific for the fecal group (positive test) and (negative reaction) for the non-fecal group of coliform bacteria, but was unsatisfactory because of its low sensitivity.

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* Material in this chapter was published previously in *Principles and Applications in Aquatic Microbiology* (Reference 136).

Howe(*12*) was one of the first investigators to apply methods of statistical analysis to his coliform data. He reported isolation of 630 strains from human feces that were, according to his observations, typical *Escherichia coli*. There was no statistical correlation in this group of *E. coli* between the presence or absence of motility, fermentation of dulcitol or mannitol, reduction of nitrates, or indole production. These data caused Howe to recognize only the sucrose-positive *E. coli-communior* and the sucrose-negative *E. coli-communis*.

Smith(*13*), in one of his original papers, observed that *B. coli* produced twice as much hydrogen as it did carbon dioxide and that *B. aerogenes* produced equal volumes of hydrogen and carbon dioxide in the fermentation of glucose. In interpreting these data, Smith failed to correct differences in solubility of the two gases in the culture medium, which resulted in the erroneous calculation of the ratios. He should, however, be credited as the first to suggest the differential characteristic of the hydrogen-to-carbon dioxide ratio.

THE GAS-RATIO (HYDROGEN-TO-CARBON DIOXIDE) DIFFERENTIAL TEST

Harden and Walpole(*14*), in carefully controlled experiments, demonstrated that the hydrogen-to-carbon dioxide ratio was 1:1 for *E. coli* and 1:2 for *Aerobacter aerogenes*. Some years later, Rogers and his group believed the gas-ratio technique could solve the problem of differentiating between coliform bacteria of fecal origin and those of plant or soil origin. Rogers, Clark, and Evans(*15, 16*), using an accurate and carefully controlled, but complicated and tedious, experimental technique, demonstrated that the fermentation of glucose by coliform strains isolated from bovine feces produced approximately equal quantities of hydrogen and carbon dioxide (1:1 ratio), while coliform strains isolated from grains produced two or more times as much carbon dioxide as they did hydrogen (1:2 to 1:3 ratios). They concluded that the low or high gas ratios gave an accurate separation between the coliform strains derived from warm-blooded-animal feces and the coliform group associated with grains. They were equally certain that the difficulties of the test and the time required for the procedure made it impracticable as a routine laboratory method. In analyzing their data, they observed an absolute correlation between the gas ratios and the terminal concentration of hydrogen ions. Clark and Lubs(*17*) found the concentration of hydrogens in the low gas-ratio strains to be in the range of pH 4.2 to 4.6, while the high-ratio strains under the same conditions produced reactions of pH 5.6 or greater.

THE METHYL RED TEST

Based on observation of the excellent correlation of gas ratios and hydrogen ion concentrations, Clark and Lubs developed the Methyl Red Test. Methyl red indicator solution was considered a suitable reagent that gave a red color at pH 4.4, changing through shades of reddish-yellow to yellow at pH 6.0. Using approximately the same medium as for the gas-ratio technique, the authors recommended a simple procedure involving the color change of an indicator solution as a practical routine laboratory procedure equal in value to the complicated gas ratio technique.

The scientific evidence for the validity of the methyl red test in the separation of the fecal from the non-fecal coliform group must be based on acceptance of the following assumptions:

1. The short series of coliform strains from bovine feces represents, without exception, the fecal coliform types found in the feces of all warm-blooded animals, including mankind.
2. The coliform group isolated from the grain samples likewise represents all coliform bacteria of non-fecal origin.
3. The "absolute correlation" between the gas ratios and the terminal concentration of hydrogen ions (pH value) would exist when the series of tests were extended in length to represent a statistically valid series.
4. The methyl red indicator test and the gas ratio test are exceptions to the observed variability of all other biochemical reactions within the coliform group.
5. The reference samples used as the fecal group and the non-fecal group do not vary with geographical distribution, climatic conditions, or other environmental factors.

If all these assumptions are true, then the methyl red test does, in fact, become a single test procedure to distinguish between the fecal and the non-fecal coliform groups of bacteria, as was concluded by Rogers and his group(*15, 16*) and specifically expressed by Clark and Lubs(*17*).

We are not able to accept the above assumptions and therefore reject the idea of the methyl red test being a reference procedure for determining the origin of the fecal and non-fecal coliform groups, although we agree it has considerable value, in a statistical way, along with other tests, in determining the sources of the coliform bacteria. Numerous investigators, including those in our own laboratory, have isolated statistically significant numbers of methyl red negative strains from human feces, but only rarely (0.6 percent) from the feces of other domestic animals. These data suggest that the choice of bovine samples by Rogers, et al., was unfortunate. The possibilities cannot be excluded that (a) the gas-ratio technique is an accurate measure of the fecal or non-fecal origin of the coliform strains that does not give an absolute correlation with the methyl red test, or (b) the gas-ratio technique would yield high ratios on the methyl red negative fecal coliform strains, which is contrary to Rogers' theory. This suggests an interesting problem for basic research the solution of which would add valuable information to a missing portion of the coliform literature.

THE INDOLE REACTION

The decomposition of tryptophane by the coliform group, depending on the enzyme systems present, may produce one or more of the following compounds in addition to other metabolites: Indolepropionic acid, indoleacetic acid, skatole, or indole. Only the property of producing indole from tryptophane has been extensively applied in the classification of the coliform group. Numerous procedures, mainly borrowed from the biological chemist, have been proposed for the detection of indole. The first was used in the early 1880's. Most of the proposed methods gave reasonably adequate sensitivity for the detection of indole, but many were unsatisfactory owing to their poor specificity, which arose from interference by the closely related chemical compounds listed above. Frequently, the interfering substances yielded positive tests in the absence of indole, which resulted in false conclusions. The exact procedure used in the detection of indole must, therefore, be included with the data, and the limitations (specificity and sensitivity) of the methodology must be considered before interpreting the results.

At least 11 different methods were recommended for indole detection between 1880 and 1920. The 11 procedures reviewed by the authors do not represent all of the technical literature on the subject. The various methods reviewed suggest considerable differences in opinion existed among the investigators. Fellows and Clough(*18*) published an excellent review of the earlier methods with references for those interested in tracing the development of the various indole reactions used in bacteriology.

Recommended Indole Test Procedure

Exact standardization of the indole test procedure is essential to secure acceptable and comparable data from different laboratories. The indole test described in *Standard Methods*(*19*) is the method we chose. Both the sensitivity and specificity of this procedure could be improved, but changes are not recommended at this time because of its wide usage, standardization, and simplicity, and because of the quantity of data on the coliform group already accumulated by this procedure, which is a modification of the original Ehrlich Reaction. A red color is obtained with indole and an orange color with skatole; the red color masks the orange when the concentration of indole is relatively larger than the quantity of skatole. The presence of small concentrations of both compounds produces a confusing color reaction. A pink or red color occurring in the medium, which is insoluble in amyl alcohol or chloroform, is a reaction indicating indolepropionic and indoleacetic acids. The original Ehrlich reaction gave a violet-blue for skatole in place of the orange color in the *Standard Methods* test.

Discussion

Numerous methods have been used to detect indole. Indole data should only be accepted when the test procedure is known; the data should be rejected when this information is not available, because each procedure varies in sensitivity and specificity. These variations restrict comments on the indole reaction to the most general considerations; determination of their applicability in a specific procedure depends on the investigator.

Frieber(*20*) explained indole formation on the assumption that all strains of coliform were able to produce indoleacetic acid in the metabolism of tryptophane; some strains were able to produce indole from this compound (indole positive) while other strains could not (indole negative). Neisser(*21*) believed the ability or inability to produce indole was a fixed characteristic of the specific strain of coliform bacteria but that this characteristic was without taxonomic value in species classification. He apparently agreed with Smirnow that positive strains could lose the indole-producing ability in certain environments. Smirnow(*22*) demonstrated *B. coli* first lost its ability to produce indole when cultured in strong solutions of glucose (3 percent), sodium chloride (4 percent), sodium sulfate (1.5 percent), or phenol (0.25 to 0.75 percent). Fabry(*23*) confirmed the phenol report by demonstrating that indole production was lost by cultivation of *Escherichia coli* strains on 0.5 percent phenol medium. Kulp(*24*) emphasized the dependence of the data on the method used to detect indole, and he reported that every strain of bacteria listed in the literature as indole positive had also been listed as indole negative, with the single exception of *Vibrio cholera*. Current indole procedures were not satisfactory to Holman and Gonzales(*25*) or to Fellows and Clough(*18*). They recommended steam distillation of the culture as essential for removing the nonvolatile interfering substances before applying the indole test procedure to the steam distillate. They even explained, unconvincingly, that this would involve practically no extra work as a routine laboratory test.

Interpretation and Use

Coliform strains isolated from the feces of warm-blooded animals are generally over 90 percent indole positive. Geldreich, et al.,(*26*) reported 94.9 percent indole-positive strains in 4,512 coliform cultures isolated (on solid medium) from the fresh feces of humans, cows, sheep, pigs, and three species of birds. The same investigators (Geldreich et al.,(*27*)) found that 19.5 percent of the coliform group, representing 2,348 strains isolated from soils, were indole positive. These surface-soil samples were collected from various geographical areas in the United States, a sanitary inspection of which showed "no known sources of fecal pollution evident in the immediate area."

Many investigators before the 1930's used the positive indole test to indicate the fecal origin of the coliform bacteria. The basis for its acceptance rested on the frequency of the presence of indole-positive strains of coliform bacteria in locations where fecal pollution was known to exist and the reported infrequency of the positive strains in the absence of pollution. Its use in recent years has generally been restricted to one of four tests for IMViC types. Currently, some investigators have suggested the use of a selective procedure, frequently an elevated incubation temperature plus the indole-positive test, to determine *E. coli*, type I, or the fecal coliform group. Procedures in this general category have been proposed by Delaney, McCarthy, and Grasso(*28*), Buttaiux, Samaille, and Pierns(*29*), Papavassiliou(*30*), Veger(*31*), and by others.

Summation

The positive indole test for coliform bacteria from warm-blooded animal feces is generally in excess of 90 percent. The hypothesis of considering indole production as an exclusive characteristic of the fecal coliform group must be questioned, however, because approximately one-fifth of the coliform bacteria from unpolluted soils can produce indole.

THE VOGES-PROSKAUER (ACETYLMETHYLCARBINOL) TEST

Voges and Proskauer(*32*) noted a color reaction when a few drops of a strong solution of potassium hydroxide was added to certain bacterial cultures that had been grown in peptone glucose broth. Some species of bacteria produced a substance that reacted under these conditions to give a red, fluorescent color, while the other species failed to give a characteristic reaction. They recommended the test, which they named the "Voges-Proskauer Reaction," as having diagnostic value as a biochemical test in bacterial identification.

Hardin and Walpole(*14*), in their studies on the fermentation of glucose by the coliform group, found a crude glycerol that they identified as 2,3-butyleneglycol ($CH_3.CHOH.CHOH.CH_3$). They presented circumstantial evidence indicating that *A. aerogenes* was able to produce, from this compound, acetylmethylcarbinol ($CH_3.CHOH.CO.CH_3$), which was in turn converted to diacetyl ($CH_3.CO.CO.CH_3$) in the presence of a strong solution of potassium hydroxide. This complex reacted with the peptone or some fraction of it to give, on oxidation by the air, the characteristic pink, fluorescent color. They explained that the absence of the reaction with *E. coli* arose from the inability of this organism to produce acetylmethylcarbinol from 2,3-butyleneglycol. The need for the peptone in the series of chemical reactions was demonstrated by pure acetylmethylcarbinol and potassium hydroxide yielding negative reactions in the absence of peptone, and positive reactions when peptone was added.

Hardin and Norris(*33*) showed that the substitution of arginine, creatinine, dicyanamide, or guanidine acetic acid for the peptone in the medium would give various shades of pink color; the fluorescence was always absent, however, which emphasized the need to read both fluorescence and color in the test. They also demonstrated that carbohydrates such as fructose, mannose, galactose, arabinose, or adonitol could be substituted for the glucose. Hardin and Norris proposed that the name of the reaction be changed from the "Voges-Proskauer Test" to the "Acetylmethylcarbinol Reaction" to distinguish it from the original procedure, which was a reaction for hemorrhagic septicemia.

Various procedural changes were suggested. West(*34*) increased the speed of the reaction without loss of specificity or sensitivity by passing air bubbles through the alkaline medium to increase oxidation. Werkman(*35*) confirmed the work of Hardin, et al., on the chemistry of the reaction and recommended addition of a catalyst to increase oxidation rate (ferric chloride (2%), two drops). His most important contribution was, however, the emphasis he placed on adoption of a standardized technique for the reaction. Both Paine(*36*) and Williams and Marrow(*37*) showed that all members of the coliform group could produce acetylmethylcarbinol during growth, but that the negative strains could decompose the compound as rapidly as it was produced.

Recommended Test Procedure

The procedure for the detection of acetylmethylcarbinol (The Voges-Proskauer Test) described in *Standard Methods*(*19*), is the method recommended.

Interpretation and Use

The production of acetylmethylcarbinol from the fermentation of glucose is not a characteristic peculiar to the coliform group, but may be detected for a number of species and several genera of bacteria. It is produced by a number of the aerobic spore-producing and non-spore-producing bacteria found widely distributed in soils.

Durham(*38*) stated that the reaction would separate the negatively reacting *E. coli* from the positively reacting *A. aerogenes* strains. Levine, et al.(*39, 40, 41*), and others have interpreted a negative reaction as indicating that the coliform bacteria were probably derived from fecal sources and that positively reacting strains were probably from grains and unpolluted soils. Others have questioned this interpretation. Originally, acceptance of the test was based on the high degree of negative correlation where fecal pollution was expected and the high positive correlation in samples believed free from fecal pollution. In later years, the proponents of the Voges-Proskauer test have used their favorite comparative test, e.g., the citrate utilization test, to establish its validity, while the critics have used comparative data with the methyl red or the indole reactions. In most of these studies, a limited series of coliform cultures derived from a single geographical area (local samples) was used. All these data should be re-examined and only those accepted in which the coliform strains were derived from known sources.

Stability of the Voges-Proskauer Test

Clark, Kabler, and Geldreich(*42*) demonstrated that Voges-Proskauer positive cultures maintained on nutrient agar slants from 6 months to 2½ years may lose their ability to produce a positive reaction; in a series of 458 coliform cultures that gave a positive reaction at the time of isolation, 105 strains or 22.9 percent lost this ability to produce a positive reaction. No explanation for this change can now be offered, but, if the theory of Paine(*36*) and of Williams and Marrow(*37*) is accepted, the positive to negative change presumably was due to the activation of the latent enzyme system. This explanation, however, is pure speculation offered without proof.

Summation

The Voges-Proskauer test or acetylmethylcarbinol reaction is useful in separation of the fecal from the non-fecal coliform group when the data are interpreted with due regard for statistical probability. The reaction is not infallible. Traditonally, it has been used as a taxonomic characteristic in the identification of the various species of the coliform group.

THE CITRATE TEST

Brown(*43*) observed that the various members of the coliform group behaved in a differential manner when grown on blood-agar plates made from citrated blood. He suggested the presence or the absence of fermentation of the citrate radical could be used as a differential procedure for the separation of *Bacterium coli* from *Bacterium aerogenes*. Koser and Rettger(*44*) started their investigation of the use of chemically defined sources of nitrogen and carbon in the cultivation of the coliform group approximately 2 years before publication of the Brown report. In a continuation of this investigation, Koser (*45*) completed work on a long series of the salts of the lower molecular weight organic acids as the sole source of carbon for the various members of the coliform group. His work confirmed the suggestion of Brown in that he became convinced that the fecal *E. coli* could not utilize the carbon source in the citrate radical, and that *A. aerogenes*, in the absence of any other source of available carbon, showed growth and fermented it and produced an alkaline reaction in the range of pH 8.4 to 9.0. In addition to the citrate radical in the form of a salt of potassium or sodium, an adequate source of nitrogen and the compounds necessary for the inorganic metabolism of *A. aerogenes* are required.

According to Koser, the commonly used reactions for distinguishing the fecal from the non-fecal strains within the coliform group were the methyl red test and Voges-Proskauer test, but a review of the work during this period indicates the indole test should also have been included. The uric acid test was also used sufficiently to receive some recognition. Koser (*46*) attempted to evaluate his citrate test by comparison with the methyl red, Voges-Proskauer, and uric acid tests. He avoided the error of many early investigators in attempting (at least to his own satisfaction) to establish the source of both the fecal and non-fecal coliform groups. The sources of the fecal coliform cultures were strains isolated from the fresh feces of warm-blooded animals while his non-fecal coliform bacteria were strains isolated from soils where he could find no evidence of fecal pollution. The conclusions from this investigation were: (a) Citrate-positive strains were characteristic of unpolluted soils, (b) citrate-negative strains were the typical fecal type of *E. coli*, and (c) the citrate reaction gave better correlation with the original sources of the coliform groups than the other tests included in the study. The data are reproduced in Table 1. Unfortunately, in the original publication the data were not presented by individual cultures for each of the four test procedures.

TABLE 1. NEGATIVE REACTIONS BY VARIOUS TESTS ON COLIFORMS FROM FECAL SOURCES AND FROM NON-FECALLY-POLLUTED SOILS[a]

Source of coliform strains	Citrate utilization	Uric acid test	Methyl red reaction	Voges-Proskauer test
Fecal strains, 118 cultures	**90.7**	**91.5**	**7.6**	**7.6**
Non-fecal strains, 72 cultures	**2.8**	**40.3**	**51.4**	**52.8**

[a] Data are expressed as the percentage of negative test reaction by each individual test. The data were not tabulated by each test for the individual strains studied (reference 46).

Recommended Test Procedure

The procedure recommended for detection of citrate utilization is the sodium citrate test as described in *Standard Methods(19)*. The slant, which indicates visible growth on the surface when the indicator color changes to blue, is easier to read than the turbidity in a colorless solution.

Interpretation and Use

The presence or absence of citrate utilization has considerable statistical value in distinguishing between the fecal and the soil type of non-fecal coliform strains. We believe it does not have sufficient specificity to be used as a single test. In Koser's data (Table 1), the citrate reaction missed 9.3 percent of the fecal coliform group and 2.8 percent of the soil types.

Summation

The citrate utilization reaction is useful for the separation of fecal and non-fecal types of coliforms on a statistical basis.

THE IMViC CLASSIFICATION

Various biochemical tests and specialized procedures such as indole, methyl red, Voges-Proskauer, and citrate utilization have been proposed as a way to correlate the relationship of the bacterial strains of the coliform group with their environment. None of these procedures was entirely satisfactory in interpreting the significance of a specific group or of an individual strain. Parr(47) studied the literature published between 1924 and 1937 on separation of fecal from non-fecal coliforms in an attempt to develop an improved classification for this group. His review indicated that during this period the investigators used the following test procedures for separation of the fecal from the non-fecal types of coliform strains:

Procedure	No. times used
Voges-Proskauer reaction	22
Methyl red test	20
Citrate utilization test	20
Indole test	15
Uric acid test	6
Cellobiose fermentation	4
Gelatin liquefaction	3
Eijkman test	2
Hydrogen sulfide production	1
Sucrose fermentation	1
a-Methyl-d-glucoside fermentation	1

On the basis of his experience and data from published reports of most other investigators, Parr chose the indole, methyl red, Voges-Proskauer, and citrate tests as the combination that would yield the best classification. The series of four reactions was designated by the mnemonic IMViC Test.* Parr reasoned that, if the four most commonly used procedures gave reasonably acceptable results when each was applied as a single test, the series would give the greatest possible accuracy. The statistical proof of this assumption is beyond the scope of this discussion. This series of four tests gives 16 possible combinations. The interpretation of these combinations was described by Parr (47) as follows:

Escherichia group: consisting of IMViC types + + − −, + − − − and − + − −, which are to be considered of fecal origin.

Aerobacter group: consisting of IMViC types − − + +, − − + − and − − − +, which probably represent the majority of the soil types.

Intermediate group: consisting of the remaining 10 possible IMViC types.

The IMViC classification for interpretation of the sources of the coliform group represented a step forward in that it was definitely superior to any of the four tests used singly and was an improvement over previously used methods.

CONDITIONS FOR ACCEPTANCE OF IMViC CLASSIFICATION

Certain assumptions were made before the validity of the IMViC classification of the coliform group could be accepted without reservation. These assumptions were:

1. Some IMViC combinations occur only in the fecal environment; other combinations are characteristic of the soil habitat; and so on for the distribution of the coliform group in nature.
2. Reactions for the indole, methyl red, Voges-Proskauer, and citrate utilization tests are unchanging for each strain of coliform bacteria in their normal habitat.
3. Marked changes in the chemical, physical, or biological environment will not produce any change in the IMViC reaction.

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* IMViC is used, with plus and minus signs, to express the differences between the organisms in a “formula.” *I* stands for indole reaction, *M* for methyl red reaction, *V* for the acetyl-methyl-carbinol test (originated by Voges-Proskauer), *i* is for euphony, and *C* for growth in mineral solution containing citrate as a sole source of carbon. As an example, an organism of the coliform group designated as “IMViC + + − −” would be *E. coli* since this gives positive indole and methyl red reactions, but negative Voges-Proskauer and citrate reactions.

Discussion

Certain reservations prevent complete acceptance of some of the assumptions listed. Correlation of the data with the first assumption was reasonably good when many strains from a single source were examined to give a statistically significant percentage, but unexplained discrepancies occurred when a decision had to be made on the results from a few coliform cultures. The second assumption must be accepted in the absence of any reliable scientific evidence that demonstrates a change in the IMViC type of any coliform strain while in its natural habitat. Laboratory experiments have changed indole-positive to indole-negative strains by cultivation on phenol medium, strong glucose solutions, and other chemicals (see indole test); have altered the positive Voges-Proskauer test to a negative reaction in the maintenance of stock cultures (see Voges-Proskauer test); or have produced temporary loss of typical colony formation, fermentation reactions, and related biochemical characteristics by cultivation on low concentrations of 2-methyl-1,4-naphthoquinone (0.0025 percent or less) as demonstrated by Colwell(*48*), all of which proves that such changes may occur in an artificial environment. No definitive proof has been presented that any of these changes in the IMViC types occur in nature, but the possibility cannot be excluded. This is especially true in waters that contain some of the organic compounds from industrial wastes or in waters high in electrolyte content. Thus, the third assumption that the sudden changes in environment will have no effect on the IMViC type of any strain of coliform must, with our present knowledge, be questioned.

Summation

The four reactions designated by Parr were the best available at the time he developed the IMViC classification of the coliform group. The fecal or non-fecal classification of the coliform group yields good results when the number of strains examined from a single source is sufficient to be statistically significant. The relationship between sources (fecal, soil, and vegetation) and types has produced a few unexplainable results, which make the interpretation of the significance of a few strains a risky procedure. Experimental proof has demonstrated that the indole and Voges-Proskauer reactions may undergo changes in an artificial environment. The possibility is suggested that these changes cannot be excluded in certain types of waste waters.

THE ELEVATED TEMPERATURE TEST

The elevated temperature test for the separation of the fecal from the non-fecal coliform groups was originally proposed by Eijkman. It was based on his findings that the coliform bacteria derived from the gut of warm-blooded animals produced gas from glucose at 46°C while the coliform strains from non-fecal sources failed to show growth. This test procedure was widely accepted in Europe and is still an approved method in some areas. The Eijkman reaction, or one of its many modifications, was studied by an impressive list of investigators, but conclusions differed concerning its sensitivity, specificity, and the interpretation of data. Perry and Hajna (*49*) proposed an elevated temperature test for *E. coli* that gained little acceptance by the bacteriologists investigating pollution. Perry and Hajna, however, improved the Eijkman conception by adding bile salts and a buffer system, and by reducing the air incubation temperature to 45.5°C, which improved the sensitivity with slight loss in specificity. The British bacteriologists used MacConkey's broth at a temperature of 44°C for the confirmation of *E. coli*, type I. Most recently, Vaughn, Levine and Smith (*50*) recommended a buffered, boric acid, lactose broth for the enrichment and identification of *E. coli;* they believed that the reduction of the incubation temperature of 43°C for 48 hours increased sensitivity and the addition of the boric acid inhibited the growth of the *Aerobacter* genus for the maintenance of specificity. We reviewed the voluminous literature on the elevated temperature reaction and tested the various procedures in the laboratory with the following conclusions:

1. The most acceptable temperature for the incubation of the fecal coliform group is 44.5°C (± 0.5), which represents, of necessity, a compromise between acceptable sensitivity and specificity for the fecal types.
2. A small percentage of the fecal coliform cultures is excluded and an equal percentage of non-fecal coliforms is included at this recommended incubation temperature.
3. Many of the usual laboratory media for cultivation of the coliform group are toxic at this elevated temperature.
4. Several media are acceptable, but the EC medium described by Perry and Hajna gives the most rapid results, requiring only 24-hour incubation as compared with brilliant green lactose bile broth that requires 72 hours to give equivalent results.
5. The test can be used only as a confirmatory procedure for coliform cultures growing on a nonselective medium.
6. The confirmatory procedure is not applicable to the microorganisms growing on a selective medium that contains inhibitory agents in its composition, e.g., Endo agar or M-Endo-MF broth.
7. The senstivity and specificity of the test should be compared on recently isolated strains from fresh feces and on coliform cultures freshly isolated from soils that were, after a careful sanitary survey, designated as having no known sources of fecal pollution.
8. In the evaluation of results, all coliforms isolated from the feces of warm-blooded animals must be considered as fecal coliform strains, and all cultures isolated from unpolluted soils must be considered as non-fecal coliform strains.

Applying the above concepts to their investigation, Geldreich, et al.,(*26, 27*) studied the coliform group isolated from the feces of a representative number of warm-blooded animals including humans, cows, sheep, pigs, chickens, ducks, and turkeys; from 223 soil samples with no known fecal pollution, collected in 26 states; and from 28 fecally polluted soil samples collected locally from feed lots or locations recently flooded with domestic sewage. The bacteriological methods used in these studies consisted of the isolation of 100 strains of coliforms from each fecal sample; purification of the strain on eosin methylene blue agar; examination by the *Standard Methods* Completed Test, for IMViC reactions, and by the elevated temperature test for production of gas in EC medium within 24 hours when incubated in a constant-temperature water bath at 44.5°C (± 0.5). The procedure followed for soils was the same except that it was not always possible to isolate 100 strains of coliform bacteria from a 10-gram sample of unpolluted soil. Data from these studies are tabulated in Table 2 and are summarized as follows:

TABLE 2. COMPARISON OF COLIFORM STRAINS ISOLATED FROM WARM-BLOODED ANIMAL FECES, FROM UNPOLLUTED SOILS AND POLLUTED SOILS BY THE IMViC REACTIONS AND THE ELEVATED TEMPERATURE TEST IN EC MEDIUM AT 44.5°C (± 0.5)

Test	Coliform strain isolated from warm-blooded animal feces, %	Coliform strains isolated from soils	
		Unpolluted, %	Polluted, %
+ + − −	91.8	5.6	80.6
+ + − −, + − − −, and − + − −	93.3	8.9	80.7
Indole positive	94.0	19.4	82.7
Methyl red positive	96.9	75.6	97.9
Voges-Proskauer positive	5.1	40.7	97.3
Citrate utilizers	3.6	88.2	19.2
Elevated temperature (FC) positive	96.4	9.2	82.9
Number of cultures studied	8,747	2,348	665

1. Coliform strains derived from feces gave good correlation with the + + − − type, the Parr fecal types (+ + − −, + − − − and − + − −), the individual tests that make the IMViC classification, and the elevated temperature test.
2. Coliform strains isolated from soils (non-fecal sources) gave good correlation with the IMViC type + + − −, with the Parr

grouping of fecal types, and with the elevated temperature test, but classification by the individual reactions was a failure.

3. Polluted soils yielded coliform data that showed good agreement among the + + − − IMViC type, the Parr fecal coliform group, and the elevated temperature tests, but, owing to the mixture of the soil types of coliforms in the pollution with the fecal types, individual reactions, such as the indole test, etc., could not be used.

Although the data are not shown in Table 2, the original publication reported that the two most prevalent IMViC types found in the soil samples from unpolluted sources were 48.1 percent − + − + type and 18.8 percent − − + + type for a total of 66.9 percent of the coliform strains present.

The data in Table 2 had to be based on the assumptions that all coliforms present in the guts of warm-blooded animals were fecal coliforms and all those found in samples of soils where no known sources of pollution could be identified were typical soil-type coliforms of non-fecal orgin. The IMViC types found in the feces of humans and chickens were especially heterogenous in their mixtures, while those of the cow, pig, and sheep were relatively uniform, with the + + − − type predominant. The possibility cannot be excluded that some of the coliform bacteria in the feces of some individuals were present by accident rather than as normal inhabitants of the gut. It is also possible that some of the soil samples calculated as unpolluted samples had actually received small quantities of fecal pollution from domestic or wild animals. We have had, however, to consider these sources as "reference samples" for the fecal and non-fecal coliform groups until better definitions can be offered.

SEPARATION OF FECAL FROM THE NON-FECAL COLIFORM GROUPS

The perfect procedure to distinguish between the coliform bacteria from a fecal environment and the coliform strains with the normal habitat in a non-fecal environment has not been discovered. Criticism of the reference samples does not, however, invalidate the use of discriminatory tests in the practical solution of pollution problems. We believe this distinction can be made by using the IMViC types and interpreting the results according to the Parr classification of fecal coliform bacteria or by using the elevated temperature test, hereafter designated as the "fecal coliform (FC) test." Both procedures give essentially equivalent results. The choice depends, therefore, on technical considerations, which greatly favor the FC test. IMViC classification should be applied only to a pure culture of coliform bacteria that has been found positive by the completed test as described in *Standard Methods*(*19*), although it has been the practice in many laboratories to type any colony that produces a typical *Escherichia* or a fish-eye *Aerobacter* colony on an eosin methylene blue agar plate. This is followed by inoculation of the pure culture in three separate media and application of four biochemical tests, namely, indole, methyl red, V-P test, and citrate utilization. Generally about 1 week of considerable labor in the laboratory is involved. Abbreviated tests consisting of a combination of two or three procedures are not acceptable for careful and accurate work. We do not agree with the recommendation of Stuart, Griffin, and Baker*(51)* that the indole and V-P tests are adequate for classifications, nor with Griffin and Stuart*(52)* that "a similar separation can be made by the citrate and V-P tests." The EC test, as applied to the multiple tube procedure for the coliform group, requires the inoculation of one additional tube of EC medium for each presumptive positive lactose (or lauryl sulfate broth) tube, and incubation at 44.5°C (± 0.5) in a water bath incubator; the final results are available within 24 hours. The procedures for the quantitative estimation of the total coliform group and the fecal coliform group are detailed in Figure 1. The fecal coliform group can also be determined by the membrane filter method. Delaney, et. al.*(28)*, recommended a procedure involving both growth and the production of indole applied to the membrane filter procedure. This procedure, described in Chapter II, proposes preparation of a membrane placed on a lactose-containing medium with aniline blue as an indicator (M-FC medium), incubation at 44.5°C (± 0.5) for 24 hours, and counting of all the blue colonies, which are the fecal coliform colonies. The total coliform colony count can be made by the usual procedure for membrane filter technique.

DEFINITIONS OF METHODOLOGY

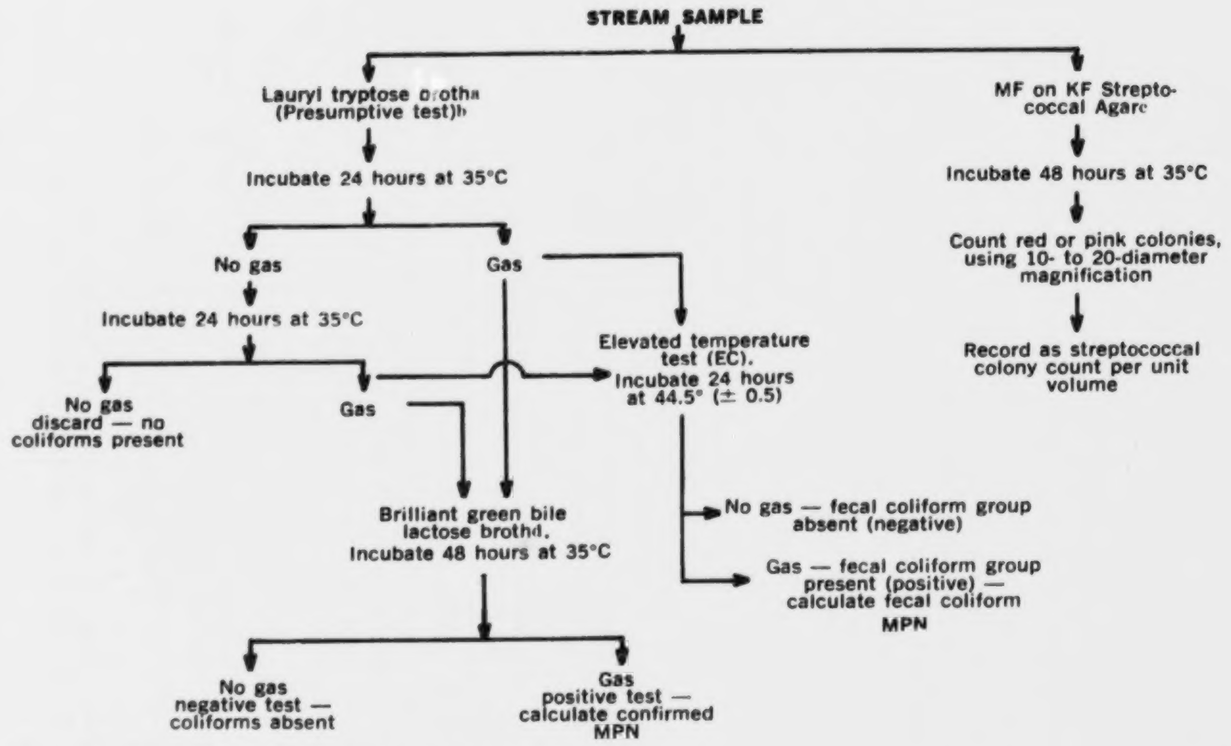
The standard tests for the coliform group described in *Standard Methods(19)* are designed specifically for the quantitative determination of the coliform group. The coliform group includes all the aerobic and facultative anaerobic, non-spore-producing, gram-negative rods which ferment lactose with gas production within 48 hours at 35°C (± 0.5). The standard tests are:

Presumptive Test (based on gas production from lactose) — A negative reaction excludes the coliform group and a postive test indicates coliform bacteria may be present.

Confirmed Test — This test demonstrates gas production from lactose in the presence of bile salts and brilliant green or the production of nucleated (with or without sheen) or opaque, mucoid, unnucleated colonies and confirms the presence of the coliform group by use of eosin methylene blue agar or Endo agar plates.

Completed Test — The coliform bacteria are isolated in pure culture, and demonstrate gas production from lactose; gram-negative rods are consistent with the morphology of the coliform group. This is the coliform reference test for the multiple-tube procedure in *Standard Methods*.

Membrane Filter Technique — The sample is filtered through a


STREAM SAMPLE
Lauryl tryptose broth[a] (Presumptive test)[b]
MF on KF Streptococcal Agar[c]
Incubate 24 hours at 35°C
Incubate 48 hours at 35°C
No gas
Gas
Count red or pink colonies, using 10- to 20-diameter magnification
Incubate 24 hours at 35°C
Elevated temperature test (EC). Incubate 24 hours at 44.5° (± 0.5)
Record as streptococcal colony count per unit volume
No gas discard — no coliforms present
Gas
Brilliant green bile lactose broth[d]. Incubate 48 hours at 35°C
No gas — fecal coliform group absent (negative)
Gas — fecal coliform group present (positive) — calculate fecal coliform MPN
No gas negative test — coliforms absent
Gas positive test — calculate confirmed MPN

[a] Lactose broth may be substituted for lauryl tryptose broth.
[b] Indicates procedure described as standard test in Standard Methods.
[c] The KF agar pour plate count may be used interchangeably with the MF colony counting technique.
[d] Eosin methylene blue or Endo agar may be substituted for the brilliant green bile lactose broth.

Figure 1 — Schematic diagram for the detection of the coliform group and the fecal-coliform group.

membrane filter, coliform colonies are cultivated on the filter surface with *Standard Methods* M-Endo medium, and a count of "coliform colonies" is made.

These procedures, because they do not distinguish between the various types of coliform bacteria composing the coliform group, yield no information as to the possible source or origin of the coliforms. A group of tentative differential tests is described, but no information is given as to their interpretation other than that such procedures seldom give sufficient information to justify the labor and time they require. Presence or absence of the coliform group is all that is necessary in the bacterial examination of a potable water, and the standard tests are adequate for this purpose.

The objectives in a bacteriological investigation of untreated surface waters, such as a stream survey, must go beyond the procedures described in *Standard Methods*. Bacteriological objectives include the quantitative estimation of all indicator microorganisms of pollution so that as much information as to the probable sources and significance can be collected as is consistent with accepted bacteriological research methods and practices.

To accomplish these objectives, newer research methods that are beyond the scope and objectives of the procedures in *Standard Methods* must be used. The testing methods include the elevated temperature test, which indicates the fecal or non-fecal origin of coliform bacteria, and the streptococcal method, which indicates pollution. Both procedures and their interpretations have been established by detailed research investigations on the distribution of indicator microorganisms in feces as well as in other environments.

DISCUSSION OF TESTS

Stream samples are collected and examined for the coliform group according to the procedures described in *Standard Methods* (*19*). These procedures include the use of lauryl tryptose broth for a screening test and confirmation of the presumptive positive tubes in brilliant green bile lactose broth, which is considered preferable to the lactose broth option in the presumptive test and the Endo or eosin methylene blue plate options in the confirmed procedure. The "Confirmed Test" enumerates the coliform group in the sample, but yields no information as to probable source, such as whether derived from feces, soil, plants, etc.

The procedure for the Elevated Temperature Test consists of the following steps:

1. Transfer a loop of broth from the positive presumptive tube to an EC broth tube containing an inverted vial to indicate gas production.
2. Place the inoculated EC tube, within less than 30 minutes after planting, in a water bath at 44.5°C (± 0.5).
3. Incubate at 44.5°C (± 0.5) for 24 (± 2) hours in water bath.

4. Record the presence or absence of gas in the inverted vial.

The presence of gas in the inverted vial is a positive reaction indicating that coliforms derived from the gut of warm-blooded animals (fecal coliforms) are present in the sample. Absence of gas indicates the absence of fecal coliforms in the tube since the members of the coliform group derived from non-fecal sources generally fail to produce gas in this medium at 44.5° C(± 0.5). The fecal coliform density is calculated on the number of positive and negative tubes in the series in the same manner as for the coliform group MPN.

SUMMATION

Bacterial species discovered in 1880 were believed to be characteristic of the bacterial flora of human feces. These microorganisms were important in health and sanitation as an indicator of fecal pollution in water and food. Microorganisms with similar morphological and biochemical characteristics were discovered in soils. Questions arose as to which species represented fecal pollution and which were native to soil, vegetation, etc. All these species with similar characteristics became known as the "coliform group" after passing through a number of transitional names and classifications. Early investigators used all known physical and biochemical tests, with considerable emphasis on the fermentation of carbohydrates, to separate the coliform group into various species. Detailed subdivisions of the coliform group into genera and species resulted but had little significance from a public health point of view because it was impossible to secure agreement on the biochemical differences between coliforms from a fecal environment and those from non-fecal habitats. Certain significant contributions were made during this time, such as the addition of an indicator of acid production to the medium by Wurtz; the inverted vial for gas production by Dunham; a theoretical basis for the bacteriological examination of water by Smith; and development of technical methods for use in sanitary bacteriology by numerous investigators.

One important objective of those concerned with water pollution continued to be the elucidation of the relationship between members of the coliform group and their natural environment. Rogers and his group believed the fecal group of coliforms and the coliforms indigenous to grains could be separated on the hydrogen-to-carbon dioxide gas ratio, and developed the methyl red test from their experimental data. Other commonly used tests with improved techniques were the Eijkman test (in Europe), indole, Voges-Proskauer, and citrate tests. Numerous other procedures were used infrequently by other investigators. Each of these procedures was believed by the respective investigators to adequately distinguish between the fecal and non-fecal groups of coliform bacteria. Parr proposed the combination of the indole, methyl red, V-P, and citrate tests as the IMViC test and defined the *Escherichia* group as being of fecal origin, the *Aerobacter* group as representive of soils, and the Intermediate group as consisting of the 10 remaining types of no defined origin.

A modified elevated temperature test was used as a confirmatory procedure to identify the fecal coliform group. This procedure was compared with the IMViC reaction on coliform bacteria from numerous sources. The elevated temperature test for distinguishing between fecal and non-fecal coliform bacteria gave results comparable to any known method, including the IMViC typing procedure. It was superior to other procedures in simplicity of technique. The perfect test for distinguishing between coliform strains of fecal and non-fecal origin has not been developed. This may be due to the difficulty of obtaining coliform bacteria from known sources for evaluation of the test procedure; to limitation of methods; or to lack of biochemical uniformity among the coliform groups. This criticism is, however, only of investigational importance in the research laboratory, since the methodologies for distinguishing these two groups are adequate for pollution investigation and the identification of fecal pollution in waters.

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CHAPTER II. FECAL COLIFORM GROUP — TECHNIQUES

Fecal coliform bacteria in water may be detected by either the multiple-tube procedure or by the membrane-filter method. In most instances the membrane filter technique will be chosen. Many bacteriologists have found this procedure to have a high degree of reproducibility, a capability of large sample-volume examination, and rapidity in obtaining data within 24 hours. Occasionally, water samples will have either a high density of particulate material, such as clay, colloidal iron, and algae, or an extremely high density of non-fecal coliform bacteria with essentially no coliforms present. Such waters are not suitable for examination by the membrane filter technique; the multiple-tube test should be used. Either the boric acid lactose broth (BALB) procedure or the *Escherichia coli* (EC) broth procedure may be used in the confirmatory multiple-tube method.

Although these two confirmatory techniques give essentially identical results, the EC procedure requires only an additional 24-hour incubation at 44.5°C as contrasted to 48-hour incubation at 43°C for the BALB method. In this chapter, the BALB procedure, the EC broth procedure, and the membrane-filter technique for fecal coliform detection are described.

MULTIPLE TUBE PROCEDURE*

Boric Acid Lactose Broth

Several methods have been suggested for the accurate, rapid, and convenient detection and enumeration of *Escherichia coli* in potable waters, stream samples, sewage, and in certain food products. The first of these procedures was described by Eijkman who proposed a "fecal coli" test that he believed differentiated between coliforms from the gut of warm-blooded animals and strains originating from cold-blooded ones. The method was based on the assumption that only fecal types of coliforms from warm-blooded animals could grow in glucose broth at 46°C with the production of gas. Twenty-five years later a close relationship between the positive Eijkman reaction and indole production was observed by Leiter (53), who suggested that the combination of the two positive tests was practically specific for *E. coli*.

Many procedures have been proposed by investigators who claim they provide a most suitable environment for the growth of *E. coli* and at the same time suppress to a large degree the growth of other coliform types along with the majority of noncoliform bacteria. Most of the procedures have required the addition of one or more selectively inhibitory substances to lactose broth with some peptone derivative and have used an incubation temperature with a narrow tolerance between 43° and 46°C. Such procedures usually were recommended as a "direct test" with the original sample inoculated into the selective medium, without any preliminary enrichment. Most investigators considered that their positive tubes represented quantitative data with the multitple tube (MPN) procedures. Opinions differed concerning the productivity of the selective media, the chief criticism being the possible failure to secure growth when minimal numbers of coliforms were planted, as in the MPN method. At the same time, the sensitivity of the medium was confused with the specificity of the reaction.

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* This material was published previously in *Applied Microbiology* (Reference 63).

The specificity of these procedures should be related to the positive reactions with one or more IMViC types of coliforms and is quite distinct from the quantitative recovery rates based on minimal number of coliforms. Other considerations that may have contributed to differences in observations between investigators were development and evaluation of methods with laboratory strains of coliforms and the differences in bacterial flora from one geographical area to another.

The reactions of the boric acid lactose broth (BALB) test described by Vaughn et al.(*50*), and Levine et al.(*54*) are compared below with strains of each coliform IMViC type as they appeared in polluted surface waters in 14 geographical areas of the United States.

Materials and Methods

Coliform organisms were isolated from samples of untreated surface waters from the Missouri River at Omaha, Nebr, and Kansas City, Kan; the Mississippi River at Minneapolis, Minn, Quincy, Ill, and Chalmetter, La; Elm Fork River near Dallas, Tex; the Rio Grande at El Paso and Laredo, Tex; the Schuylkill River at Philadelphia, Pa; Big Cottonwood Canyon at Salt Lake City, Utah; Colorado River aqueduct water at LaVerne, Calif; the Detroit River at Wyandotte, Mich; the Chattahootchee River at Atlanta, Ga; and Oradell Reservoir at New Milford, N.J.

Cooperating laboratories at each location filtered appropriate quantities of surface water through membrane filters and placed the filters on preservative (benzoated Endo) medium(*55*) in 1-ounce sterile ointment tins. They then mailed them to the Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio, where tests were completed as described by Geldreich et al.(*55*) for the delayed incubation test for coliforms. Between 100 and 200 coliform colonies were isolated in a series from the membranes from each location; generally, there were three series at regular intervals. The coliforms were immediately identified and classified by IMViC types according to the procedures described in *Standard Methods*(*56*).

Simultaneously, the BALB test devised by Levine et al.(*54*) was used as a confirmatory procedure on each coliform strain. Any noncoliform bacteria that may have been picked up in the isolation procedures were discarded as they failed in confirmatory or identification tests. The technical methods recommended by Levine et al. were followed as exactly as possible. The medium was prepared from dehydrated boric acid broth, sterilized as recommended by Vaughn et al.(*50*), and the hydrogen ion concentration of each sterile lot determined on a Beckman pH meter, model H-2. The tubes usually were inoculated from 24-hour coliform cultures and were immediately placed in a constant-temperature water bath with the water height above the level of the medium inside the tubes. Temperature of the water bath was 43°C with less than 0.5° variation and was regularly checked with a Bureau of Standards certified mercury thermometer.

Inverted vials in the boric acid lactose broth were examined for gas at 24- and 48-hour periods. The presence of gas in any quantity in the inverted vial within a 48-hour incubation period was recorded as a positive reaction, and the absence of gas in the same time was considered as a negative result. All tubes were discarded at the end of incubation for 48 hours.

Results

During this study, 4,423 strains of coliforms were examined by the boric acid lactose broth test and classified as to IMViC types. Between 216 and 450 coliform strains were examined from each sample area. Almost one-third were − − + + IMViC type, approximately another third were − + − + and + + − − types, and the remaining were distributed among nine IMViC types. Data on the types studied are summarized in Table 3.

TABLE 3. REACTIONS IN BORIC ACID LACTOSE BROTH (BALB) OF COLIFORM IMViC TYPES

IMViC type	No. of strains examined	Percentage of coliforms isolated	Positive BALB tests		Negative BALB tests	
			No. of strains	Percentage positive	No. of strains	Percentage negative
− − + +	1,414	32.0	284	20.1	1130	79.9
− + − +	764	17.3	41	5.4	723	94.6
+ + − −	731	16.5	682	93.3	49	6.7
− + + +	384	8.7	33	8.6	351	91.4
+ + + +	332	7.5	9	2.7	323	97.3
+ + − +	323	7.3	37	11.5	286	88.5
+ − + +	203	4.6	9	4.4	194	95.6
− + − −	139	3.1	21	15.1	118	84.9
+ + + −	74	1.7	60	81.1	14	18.9
− + + −	31	0.7	0	0.0	31	100.0
− − + −	20	0.5	0	0.0	20	100.0
− − − +	8	0.2	6	a	2	a
Total	4,423		1,182	26.7	3,241	73.3

a = Insufficient number of cultures studied.

In percentage of positive reactions in boric acid lactose broth, the + + − − type was highest with 93.3 percent of the strains tested. Next in descending percentage of positive reactions were + + + − type with 81 percent; − − + + with 20 percent; − + − − type with 15 percent; and + + − + type with almost 12 percent. The BALB positive strains in the next seven types varied between 8.6 percent for − + + + type to none for the − + + − and − − + − types.

A comparison by sample points of the BALB positive reactions for + + − − type with the BALB negative reactions for the remaining IMViC types is made in Table 4.

Of the total 731 + + − − type coliform strains examined, 682 or 93.3 percent were positive. Positive reactions varied between 83.8 percent at location 8 and 100 percent at location 11. For the remaining 3,692 IMViC types of coliforms (excluding + + − − type), negative reactions resulted with 3,192 or 86.5 percent of the strains examined. The percentage of negative reactions also varied among locations; location 9 was lowest with 60.7 percent and location 13 was highest with 100 percent.

Table 5 presents the number of strains examined and the percentage of positive reactions by each IMViC type for the individual sample areas. The marked variations of type reactions among sample areas are emphasized. For example, the − − + + type was 48 percent positive at location 9; 23 to 26 percent positive at locations 4, 6, and 11; and 1 percent or less at locations 7 and 13. The − + − + type was positive with 22 and 23 percent of the strains at locations 9 and 10, but had only 2 percent positives at locations 1, 2, and 8, and none at locations 4, 6, 12, and 13. The same variation can be observed for each IMViC type with the possible exception of the + + + − type. In the small number of strains examined for this type, the proportion of positive reactions appeared to be about the same in each location where they were present.

Discussion

The + + − − type gave a reasonably good percentage of positive reactions with the BALB test in many locations, but the large number of positive reactions with such types as − − + +, + + − +, and − + − − strains introduces a considerable classification error in the overall application of this test to accurately detect + + − − IMViC type alone. The latter three IMViC types represented 42.4 percent of the coliform strains isolated from polluted surface waters. The + + + − type yielded 60 positives or 81 percent of 74 strains, but as a reasonably rare type representing only 1.7 percent of the total coliform strains in these waters, it would scarcely introduce sufficient error to change the interpretation of a series of results.

Application of the tentative coliform group classification described in *Standard Methods*(56) would result in the following data: Based on the assumption that *E. coli* includes types + + − − or − + − −,

TABLE 4. COMPARISON OF POSITIVE TESTS WITH + + − − TYPE AND NEGATIVE TESTS WITH THE REMAINING TYPES (EXCLUDING + + − − TYPE) BY SAMPLE LOCATIONS[a]

Location	+ + − − IMViC type				Other IMViC types (excluding + + − − type)			
	No. of strains	No. of positive tests	Percentage positive tests	Order[b]	No. of strains	No. of negative tests	Percentage negative tests	Order[b]
1	44	41	93.2	7	230	210	91.3	4
2	28	24	85.7	12	422	366	86.7	6
3	22	19	86.4	11	275	254	92.4	2
4	81	76	94.8	5	199	179	90.0	5
5	61	59	96.7	4	194	166	85.6	10
6	72	71	98.6	2	282	243	86.1	8
7	69	65	94.2	6	147	122	83.0	11
8	37	31	83.8	13	302	287	95.0	1
9	81	75	92.6	9	326	198	60.7	13
10	60	53	88.3	10	171	147	86.0	9
11	49	49	100.0	1	383	310	80.9	12
12	55	51	92.7	8	203	175	86.2	7
13	4	2	[c]		285	285	100.0	
14	68	66	97.1	3	273	250	91.6	3
Total	731	682	93.3		3,692	3,192	86.5	

[a] Assignment of numbers to sample locations was arbitrary.

[b] Represents position of data arranged in a descending series by percentages.

[c] Insufficient number of cultures studied.

TABLE 5. NUMBER OF STRAINS EXAMINED AND PERCENTAGE OF POSITIVE STRAINS FOR EACH IMViC TYPE BY INDIVIDUAL SAMPLE POINTS

IMViC type		Sample points														Totals
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
− − + +	No. strains	84	129	164	31	113	107	76	40	201	79	146	97	16	131	1,414
	Percentage	13	16	10	23	17	25	1	17	48	14	26	15	0	11	20.1
− + − +	No. strains	41	92	35	21	27	38	21	117	40	31	92	34	149	26	764
	Percentage	2	2	6	0	7	0	5	2	22	23	15	0	0	4	5.3
+ + − −	No. strains	44	28	22	81	61	72	69	37	81	60	49	55	4	68	731
	Percentage	93	86	86	94	97	99	94	84	93	88	100	93	a	97	93.3
− + + +	No. strains	33	30	12	59	2	23	12	37	37	10	47	19	27	36	384
	Percentage	0	7	8	0	a	9	0	3	24	20	23	16	0	3	8.6
+ + + +	No. strains	21	32	17	37	9	55	8	25	14	20	43	17	1	33	332
	Percentage	0	9	6	0	a	2	a	0	7	0	0	0	a	0	2.7
+ + − +	No. strains	25	76	13	25	21	27	13	37	20	12	21	13	b	20	323
	Percentage	12	3	8	4	9	4	100	3	30	17	9	8		5	11.5
+ − + +	No. strains	19	17	30	13	18	18	6	32	2	11	12	7	9	9	203
	Percentage	5	6	0	15	11	5	a	3	a	0	8	a	a	a	4.4
− + − −	No. strains	2	18	2	7	1	9	5	6	8	4	8	5	62	2	139
	Percentage	a	5	a	a	a	a	a	a	a	a	a	a	0	a	15.1
+ + + −	No. strains	4	25	b	6	b	3	5	2	3	2	7	7	2	8	74
	Percentage	a	88		a		a	a	a	a	a	a	a	a	a	81.8
− + + −	No. strains	b	1	b	b	b	b	1	1	1	b	1	4	17	5	31
	Percentage		a					a	a	a		a	a	0	a	0.0
− − + −	No. strains	1	1	b	b	2	2	b	2	b	2	6	b	1	3	20
	Percentage	a	a			a	a		a		a	a		a	a	0.0
− − − +	No. strains	b	1	2	b	1	b	b	3	b	b	b	b	1	b	8
	Percentage		a	a		a			a					a		a(25.0%)

a Insufficient number of cultures studied.
b None.

the number of strains in this study would be 870 with 703 positive reactions, or 80.8 percent. With the same classification, the "commonly designated source" of the remaining IMViC types is usually considered non-fecal strains. In this group, 3,553 strains gave 475 (13.4 percent) BALB positive reactions. Even with this liberal tentative classification of "usual sources," the reaction does not appear sufficiently precise for group separation on the basis of common fecal or non-fecal types.

Comparison of locations by positive-reaction percentages for + + − − type with negative-reaction percentages for remaining types as listed in Table 4 reveals a peculiar distribution. Locations 14, 4, 12, and 10 appear in similarly relative positions for positive reactions with + + − − type and for negative reactions with remaining types when examined by percentages in descending order. There is marked disagreement in relative positions for the remaining locations, e.g., location 11 at the top of the series for + + − − type and next to lowest for the remaining types, and location 6 in second and eighth positions, respectively. Further disagreement in results by locations is shown in Table 5. The − − + + type gave 48 percent positives at location 9; between 23 and 26 percent at locations 4, 6, and 11; and 1 percent or less at locations 7 and 13. IMViC type − + − + gave more than 20 percent positives at locations 9 and 10, and 2 percent or less at locations 1, 2, 4, 6, 8, 12, and 13. Similar variations in percentages of positives by locations were evident in all the types investigated except + + + −, − + + − and − − + −. This lack of uniformity in data by individual locations suggests differences in the coliform flora found in waters and may account for some controversial data and interpretations. We cannot eliminate the possibility that the IMViC system of classification does not adequately divide the coliforms found in waters into groups consistent with their common sources.

As a result of these data, the BALB procedure is not recommended for accurate and rapid classification of the + + − − IMViC type. The method appears applicable for survey work, however, where an approximate value for the + + − − coliform type would meet the need. In this investigation, 4,423 coliform strains gave 1,182 positive BALB reactions, or 26.7 percent, when BALB was the only test considered. By IMViC classification, 731 strains of the + + − − type, or 16.5 percent, were present. Inclusion of the indole reaction, which has been used by Hoather and Dewey(57), Thomas et al.(58), and Bicknell, Middleton, and Neblett(59) by direct inoculation of a suitable medium from positive presumptive tubes, would greatly increase accuracy in survey procedures. By a combination of a positive indole test and a positive BALB reaction, the total positives (from Table 3) would be 797 strains, or 18.0 percent. Of course, this total does not include the 49 BALB negative strains of + + − − type or eliminate the 115 positive strains in types + + + +, + + − +, + − + +, or + + + −.

In the evaluation of these data, all comparisons were made of results from tests on freshly isolated strains with a minimum of cultivation on laboratory media so that the results might be applicable for survey work and practical water bacteriological investigations. Some reactions might change after continued cultivation and stabilization on laboratory media in a controlled environment.

Summation

Coliforms from 14 geographical areas of the United States were divided into 12 types by their IMViC reactions. The 4,423 strains were examined for their reactions in boric acid lactose broth (BALB), which was incubated at 43°C for 48 hours. There were 682 positive cultures (93.3 percent) in the 731 strains of the + + − − type, and 500 positive cultures (13.5 percent) in the 3,692 strains belonging to the remaining 11 types. Because of lack of sensitivity (6.7 percent negative reactions) for the + + − − type and poor specificity (13.5 percent positive reactions) for the other IMViC types, the BALB test is not recommended as a single specific test for the accurate detection of + + − − IMViC type.

The data vary widely by locations, however, and even at each location the sensitivity and specificity may not agree. These variations between sample areas may account for the controversial data and interpretations for this and other methods for the detection of + + − − IMViC types. Possibly, these variations may result from differences in coliform flora for which the IMViC system of classification is not adequate.

If the BALB procedure had been used as a single test, 1,182 positive reactions or 26.7 percent would have been considered as + + − − type although only 731 strains or 16.5 percent were actually + + − − by IMViC classification. The BALB test alone could be used in survey work where an approximate value for this IMViC type is sufficient. Use of both a positive indole and positive BALB test as a survey procedure, however, would not increase the time required and, by elimination of 385 false reactions, the positives would be reduced to 797 strains or 18.0 percent of the 4,423 strains tested.

EC Medium*

Several research workers have suggested that *Escherichia coli* would be a more accurate indicator of fecal contamination than the more inclusive "coliform group." Media and procedures for the enumeration of *E. coli* have been proposed by Eijkman(*11*), Leiter(*53*), Perry and Hajna (*49, 60*), Hajna and Perry (*61, 62*), Vaughn et al.(*50*), and Levine et al.(*54*). Some of these procedures apparently yield satisfactory results with certain samples of water, milk, or food products; however, there is no universal agreement as to their criteria of application and significance of results in water bacteriology.

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* This material was published previously in *Applied Microbiology* (Reference 70).

Also, uncertainties exist regarding the significance of *E.* coli when assayed by rapid tests as compared to the traditional IMViC classification.

The investigation reported here was undertaken to determine the reactions of 12 IMViC types of coliform bacteria in EC medium after 24-hour incubation at 44.5°C. We believed the resultant data would be of value in choosing the procedure and interpreting test results for *E.* coli and the coliform group.

Materials and Methods

The coliform group of bacteria for this investigation was defined as aerobic and facultative anaerobic gram-negative, nonsporeforming bacteria that fermented lactose with gas formation in 48 hours or less when incubated at 35°C(*56*). All cultures were recently isolated from surface water samples obtained from various locations with varying degrees of pollution(*63*). The tentative methods described in *Standard Methods* were used for indole, methyl red, and Voges-Proskauer tests, and Simmons' citrate agar(*64*) was used for the citrate test. The EC medium of Hajna and Perry(*62*) was used as a confirmatory test for the various coliform bacteria. The medium was reconstituted from the dehydrated product, tubed with an inverted vial for the observation of gas production, and sterilized in an autoclave at 121°C for 15 minutes.

Each culture of the coliform bacteria was examined for indole and acetyl methyl carbinol production, methyl red reaction, utilization of citrate, and gas formation in EC medium within 24 hours at 44.5°C in a constant temperature water bath. In a reading of the EC tubes, the presence of gas in any quantity was considered a positive reaction, and absence of gas (after 24-hour incubation) was a negative reaction.

This procedure differed from that recommended by Perry and Hajna(*49*) in that they suggested air incubation at 45.5°C for 48 hours.

Results

We examined 5,794 cultures of coliform bacteria from untreated surface water supplies of 14 treatment plants. Of the 1,358 that included *E. coli*, 83.7 percent showed positive EC reactions. When the *E.* coli strains were subdivided into varieties I and II (Table 6), the percentages of positive results were 92.7 and 21.8, respectively. The remaining 10 IMViC types yielded 7.8 percent positive tests for 4,436 cultures. The data summarized in Table 6 also show that type + + + − gave 77.2 percent positive reactions.

Discussion

With 92.7 percent of the *E.* coli variety I (+ + − −) showing positive EC reactions, there is relatively good agreement in the classifica-

TABLE 6. REACTIONS OF COMMON COLIFORM IMViC TYPES IN EC MEDIUM AT 44.5°C IN 24 HOURS

IMViC type	Commonly designated source[a]					
	Fecal			Non-fecal		
	No. of cultures examined	Positive EC reactions No.	%	No. of cultures examined	Positive EC reactions No.	%
+ + − −[b]	1,184	1,098	92.7			
− + − −[c]	174	38	21.8			
+ + + −				79	61	77.2
+ + − +				425	52	12.2
− − + +				1,686	179	10.6
− − + −				24	1	4.2
+ − + +				237	8	3.4
− + + +				485	16	3.3
+ + + +				453	10	2.2
− + + −				50	1	2.0
− + − +				989	18	1.8
− − − +				8	2	—
Total	1,358	1,136	83.7	4,436	348	7.8

[a] According to Reference 56.
[b] Variety I.
[c] Variety II.

tion of this organism. Only 21.8 percent of the *E. coli* variety II (−+−−) however, reacted positively in EC medium, thus reducing the correlation for the two *E. coli* varieties collectively to 83.7 percent. The IMViC type + + +− comprised only a small part of the cultures studied, but showed 77.2 percent positive EC tests. The reactions of *E. coli* variety II and of + + + − type to the EC test at 44.5°C suggest the need for further work to determine the fecal or non-fecal origin of these organisms. The latter type (+ + + −) appears to be more closely related to *E. coli* variety I on the basis of growth with production of gas at 44.5°C than does the indole negative variety II. These results are comparable to those reported by Clark et al.(*63*), who used a boric acid lactose broth reaction at 43°C. Both the boric acid lactose broth and the EC procedures seem to have approximately the same capabilities in differentiating the coliform group.

To establish the value of any single type of coliform bacteria as a sanitary indicator of fecal pollution was not the purpose of this study. The results reported, however, strongly suggest that added efforts should be made to locate sources of fecal pollution of waters that contain EC positive coliforms. Because other biochemical characteristics may show better correlations with fecal or non-fecal origins of the coliform group, further consideration should be given to the use of other measurements in addition to the conventional IMViC reactions and growth at elevated temperatures.

Summation

Coliform cultures of 12 IMViC types were incubated in EC medium in a water bath for 24 hours at 44.5°C (± 0.3). Positive tests were recorded for 92.7 percent of *Escherichia coli* variety I, for 21.8 percent of *E. coli* variety II, and for 83.7 percent when considered together as coliform bacteria commonly designated as of fecal origin. The non-fecal types of coliform cultures showed 7.8 percent positive tests. A high percentage of $++\,+-$ strains grew at 44.5°C, which suggests a close relationship to the *E. coli* variety I.

Subsequent research reported in later chapters substantiates that the BALB and EC procedures are not specific for any one type or group of coliform types. Both elevated temperature procedures were specific, however, for coliforms derived from warm-blooded animal feces. Thus, the elevated temperature test was therefore, a fecal coliform test and not an *E. coli* type $++--$ test exclusively.

MEMBRANE FILTER PROCEDURES*

Several tests in which the elevated-temperature reaction is used have been proposed for the separation of the fecal coliform group that is derived from the gut of warm-blooded animals and the non-fecal-coliform group that originates from various other sources(*26, 27, 63, 65, 66*). These elevated-temperature tests, however, have been restricted to a confirmatory multiple-tube most probable number (MPN) procedure with liquid medium.

Improved Techniques

With increasing use of the membrane filter (MF) total-coliform procedure for water pollution studies, a need has developed for a similar MF procedure in which the elevated-temperature reaction is used. Taylor, Burman, and Oliver(*67*) proposed a 2-hour enrichment on nutrient broth at 37°C followed by MF transfer to a modified MacConkey broth for incubation at 44°C for 16 hours. The fecal coliform colonies were differentiated from other organisms by their yellow color. Delaney, McCarthy, and Grasso(*28*) reported success with a tryptose bile agar (TBA) medium combined with an indole test. In this method, the MF is placed on the TBA medium and incubated 20 to 24 hours at 44.5°C; thereupon, the MF is transferred to an absorbent pad saturated with indole reagent. The *E. coli* Type I colony becomes a dark-red color upon reaction with this reagent. The necessity of either an incubation temperature transfer or an indole reagent test transfer has handicapped the use of this method in field laboratory investigations.

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* This material was published previously in *Journal American Water Works Association* (Reference 149).

Recently, a method was developed that allows the direct quantitative enumeration of the fecal coliform group by the MF technique without the need for prior enrichment or subsequent chemical test. A medium for fecal coliforms (M-FC) is recommended that contains no inhibitory substances for coliform bacteria and depends for its selectivity on an incubation temperature of 44.5°C (± 0.5) for 24 hours (± 2). The indicator system, aniline blue, and the sodium salt of rosolic acid were first proposed as differential indicators by Bronfenbrener, Schlessinger, and Soletsky(*68*).

Procedure

The M-FC medium contains tryptose, 1 percent; proteose peptone No. 3, 0.5 percent; yeast extract, 0.3 percent; sodium chloride, 0.5 percent; lactose, 1.25 percent; bile salts No. 3, 0.15 percent; and aniline blue, 0.01 percent. The reagent containing the sodium salt of rosolic acid is prepared by dissolving 1 gram of rosolic acid in 100 milliliters of 0.2 normal sodium hydroxide solution. To prepare the medium, the ingredients are dissolved in distilled water containing 0.01 percent rosolic acid and heated to the boiling point. The medium should not be sterilized by steam pressure. The final pH of the medium at 25°C is 7.4. Rosolic-acid salt reagent is stored at 2° to 8°C in the dark and must be discarded after 2 weeks' storage.

After momentary boiling, the medium is cooled to room temperature, and 2 milliliters are dispensed to each sterile absorbent pad in a plastic culture dish. Tight-fitting dishes are essential as they are submerged in a water bath. Waterproof plastic bags may be used to hold five culture dishes during submersion. These dishes are anchored below the water surface by inserting bags containing MF cultures between the three wire supports of a stainless-steel test-tube rack. Such racks (now used as two-compartment shelves) are then submerged in the 44.5°C water bath.

Sample quantities that would yield fecal coliform densities of 20 to 60 colonies per MF were chosen for the membrane filter tests. The samples were incubated 24 hours (± 2) at 44.5°C (± 0.5) by submerging the cultures (inverted) in a water bath. On this medium, the colonies produced by fecal coliform bacteria are blue and all other colonies are gray to cream colored. Background color on the membrane filter varies from a yellowish cream to faint blue, depending on the age of the rosolic-acid salt reagent. A rosolic-acid solution, not over 2 weeks old, is recommended to suppress more of the gray-to cream-colored colonies that are occasionally encountered in certain water floras.

Evaluation

Forty water samples from various rivers, creeks, domestic sewages, lagoons, lakes, and wells, and thirteen pure cultures of several coliform IMViC types isolated from water, soil, insects, fish, vegetation, and warm-blooded animals were used to evaluate the productivity and selectivity of the M-FC medium.

In the evaluation, fecal coliform counts obtained by the MF procedure were compared with results of the Confirmed Test MPN for fecal coliform organisms at 44.5°C (± 0.5) for 24 hours. The latter multiple-tube, elevated-temperature test showed a 96.3 percent positive correlation with coliform strains isolated from feces of warm-blooded animals(*26*).

Total coliform detection by the MF on M-Endo-MF broth and the multiple-tube "confirmed coliform test" and by the "completed coliform test," as described in the eleventh edition of *Standard Methods*(*19*), was evaluated. A comparison of these data shows similarities and differences in the two procedures for the total coliform densities in the same samples.

All fecal coliform colonies on the M-FC were verified by inoculating the blue colonies into phenol red lactose broth for enrichment and production of gas during 24- to 48-hour incubation at 35°C. All positive tubes were then confirmed by the elevated temperature test in EC broth incubated 24 hours at 44.5°C.

To evaluate the M-FC with pure cultures, various fecal and nonfecal coliform strains representing four IMViC types were chosen from different sources. Five replicate filtrations were cultured on M-FC medium and five replicate plates were poured with tryptose-glucose extract (TGE) agar for each of the thirteen pure culture strains. The TGE agar plates were incubated for 24 hours at 35°C and the MF cultures were incubated on M-FC medium for 24 hours at 44.5°C.

Results

The results of the evaluation of M-FC medium with 40 water samples are given in Table 7. These data include total coliform and fecal coliform bacteria counts obtained both by the multiple-tube method and by the membrane filter procedure. Ratios of counts between the two procedures on both the fecal and total coliform tests are used to evaluate the productivity of the new medium. These fecal coliform ratios were calculated from the direct M-FC colony counts and the MPN data of the multiple-tube, elevated-temperature tests. Total coliform ratios were based on the coliform colony count from M-Endo-MF and the "completed test" multiple-tube procedure. The minimum and maximum fecal coliform ratios for the 36 determinate values were 0.42 and 2.52, respectively. A similar comparison of the 40 determinant total coliform results demonstrated a 0.25 minimum and a 4.92 maximum ratio.

TABLE 7. EVALUATION OF RESULTS WITH M-FC MEDIUM ON VARIOUS WATERS IN OHIO

Sample		Number of coliform organisms per 100 ml						Coliform organism ratios	
		Total (MPN)		Total (MF)	Fecal (MPN)	M-FC		Tot. MF / Tot. MPN	Fecal MF / Fecal MPN
		Confirmed	Completed			Count	Verified		
Wells —	Baas	33	17	4.3	2.0	1.3	1.1	0.25	0.65
	Kilb	130	33	37	<2.0	2.8	2.8	1.12	Indt.[a]
	Gilman	278	221	215	<2.0	<0.8	<0.8	0.97	Indt.
Lakes —	Devou	109	70	36	11	8	8	0.51	0.73
	Sharon	221	94	80	<2.0	1.5	1.5	0.85	Indt.
	Burnet	1,700	1,100	380	220	300	270	0.35	1.36
	Winton	4,600	3,300	3,300	2,300	2,500	2,500	1.00	1.09
	Smith	172,000	49,000	35,000	13,000	5,500	5,500	0.71	0.42
Creeks —	4-Mile	490	141	80	49	22	22	0.57	0.45
	8-Mile	350	350	160	23	10	10	0.46	0.44
	Taylor	490	490	700	490	550	550	1.43	1.12
	Huntley	2,300	1,300	450	14	17	17	0.35	1.21
	Upper Cluff	7,000	2,210	900	490	370	370	0.41	0.76
	Berkshire	22,000	4,900	10,000	790	1,000	1,000	2.04	1.27
	9-Mile	10,900	7,900	15,000	330	550	550	1.90	1.64
	Lower Cluff	7,900	7,900	5,300	2,300	1,700	1,500	0.67	0.74
	Duck	78,000	49,000	86,000	10,900	6,000	5,400	1.76	0.55
	Mill	4,900,000	2,210,000	4,800,000	221,000	440,000	400,000	2.17	1.99
Lagoon —	Ludlow	49,000	14,100	12,000	1,720	3,600	2,900	0.85	2.09
Rivers —	Little Miami-Kellogg	1,720	1,090	2,100	330	150	150	1.93	0.46
	Beechmont	10,900	2,210	3,300	330	290	260	1.49	0.88
	Newtown	10,900	10,900	22,000	3,300	2,800	2,800	2.02	0.85
	Great Miami	840	310	470	23	58	46	1.52	2.52
	Licking	10,900	10,900	16,000	3,300	3,700	3,700	1.47	1.12

[a] Indeterminate.

TABLE 7. EVALUATION OF RESULTS WITH M-FC MEDIUM ON VARIOUS WATERS IN OHIO (CONT'D)

Sample	Number of coliform organisms per 100 ml						Coliform organism ratios	
	Total (MPN)		Total (MF)	Fecal (MPN)	M-FC		Tot. MF / Tot. MPN	Fecal MF / Fecal MPN
	Confirmed	Completed			Count	Verified		
Rivers (cont'd) —								
Ohio (mile No.)								
453.9	3,300	3,300	1,300	172	200	180	0.39	1.16
461.6	1,720	700	2,300	172	160	160	3.29	0.93
463.0	460	310	260	130	56	50	0.84	0.43
464.1	700	490	1,300	172	140	140	2.65	0.81
465.0	17,200	17,200	29,000	3,300	1,400	1,100	1.69	0.42
469.8	33,000	17,200	44,000	3,300	6,800	6,800	2.56	2.06
472.4	46,000	14,100	45,000	4,600	4,200	4,200	3.19	0.91
475.1	1,300,000	1,300,000	3,100,000	790,000	1,300,000	1,300,000	2.30	1.65
482.0	49,000	49,000	52,000	14,100	10,200	10,200	1.06	0.72
485.8	23,000	13,000	64,000	4,900	4,800	4,300	4.92	0.98
513.3	230	230	440	79	58	58	1.91	0.73
517.6	130	27	56	17	12	12	2.07	0.71
522.1	130	11	22	5	<2	<2	2.00	Indt.
Domestic Sewage —								
Linwood	13,000,000	7,900,000	19,000,000	4,900,000	5,400,000	5,400,000	2.41	1.10
Beechmont	33,000,000	17,200,000	31,000,000	4,600,000	6,400,000	5,800,000	1.80	1.39
Berkshire	54,200,000	34,800,000	35,000,000	4,900,000	5,000,000	4,500,000	1.01	1.02

Productivity of the M-FC medium was also evaluated in a study of the recovery rates for a group of pure cultures isolated from different sources. The coliform strains included + + − −, − − + +, − + − +, and + − + + IMViC types. Table 8 shows excellent correlation between the EC fermentation reaction and an average 88 percent recovery for all fecal coliform strains examined. No recovery was detected for the five non-fecal coliform strains.

Selectivity of the M-FC medium was demonstrated by biochemical studies of blue- and cream-colored colonies isolated from numerous samples.

TABLE 8. PURE CULTURE RECOVERIES ON M-FC MEDIUM

Coliform organism IMViC type	Source	EC reaction	TGE count	M-FC count	Recovery, %
+ + − −	Water	+	165	160	97
+ + − −	Water	+	89	78	87.6
+ + − −	Water	+	31	28	90.3
+ + − −	Soil	+	103	78	75.7
+ + − −	Soil	+	37	37	100
+ + − −	Warm-blooded animal	+	161	150	93.2
+ + − −	Warm-blooded animal	+	74	61	82
− − + +	Insect	+	91	71	78
− − + +	Water	0	77	<1	0
− − + +	Water	0	101	<1	0
− − + +	Soil	0	66	<1	0
− + − +	Fish	0	113	<1	0
+ − + +	Vegetation	0	64	<1	0

Results are reported in Table 9. Of 3,031 blue colonies selected for verification, 93.2 percent were confirmed as fecal coliform organisms. Because the non-fecal (cream-colored) coliform colonies were never very numerous at an incubation temperature of 44.5°C, only 460 colonies were examined for lactose fermentation at this temperature; 83.9 percent were verified as being non-fecal coliform strains.

TABLE 9. FECAL COLIFORM VERIFICATION OF BLUE AND CREAM-COLORED COLONIES ON M-FC MEDIUM

	Blue colonies			Cream colonies		
Source	Total No.	Number verified as fecal	Fecal, %	Total No.	Number verified as non-fecal	Non-fecal, %
Wells	60	53	88.3	9	9	100.0
Lakes	60	57	95.0	64	53	82.8
Creeks	496	456	91.9	114	88	87.2
Rivers	2,222	2,076	93.4	208	176	84.6
Lagoon	43	39	90.7	0	0	0
Sewage	150	143	95.3	65	60	92.3
Total	3,031	2,824	93.2	460	386	83.9

Discussion

A comparison between the MF and MPN fecal coliform procedure must be guided by several factors inherent in the methods involved. First, the MF count is based on an actual count of fecal coliform colonies detected in a given amount of water sample. The MPN is a statistical estimate of the most probable density for a given number of positive and negative fermentation tube reactions occurring in the three-dilution multiple-tube test. The 95 percent confidence limit for the five-tube three-dilution multiple-tube method yields MPN values between 31 and 289 percent of the actual bacterial density of the water sample. Thus, if a single water sample is analyzed 100 times, 95 of these MPN results will be between 31 and 289 percent of this density. The remaining 5 MPN values will be higher or lower than this range. Judged by these criteria and by a reference standard consisting of the results from 36 tests by the multiple-tube MPN method, the results obtained in parallel tests with the MF procedure were within the 95 percent confidence limit.

Second, lactose fermentation is measured in the MF procedure by the production of acids, whereas, in the MPN test, gas production is positive evidence of fermentation. Our data demonstrating that 93.2 percent of the blue colonies are fecal coliform organisms, along with the excellent correlation of reactions for the coliform bacteria in pure cultures, indicated the two test procedures were measuring predominantly the same group of organisms: the fecal coliform bacteria.

The size of the water sample to be examined by the MF technique must be carefully considered before filtration is started. The only limitations in choosing suitable quantities are the bacterial density and the amount of suspended matter in the sample. Although the filtration procedure lends itself to testing a wide range of sample volumes, the technician is restricted in his choice of sample increments for a given water examination. This restriction is illustrated for four different sources of samples in Table 10. A proportional rela-

TABLE 10. SELECTION OF SAMPLE FILTRATION VOLUMES FOR THE M-FC TEST[a]

Source of sample	Dilution, ml	Fecal coliform colony count	Calculated colony density
Little Miami River	0.1	6	4
	0.2	9	8
	0.5	21	20
	1	39[b]	39
	2	77	78
	4	150	160
	8	260	310
Linwood sewage	0.0001	4	5
	0.00025	11	10
	0.0005	25	19
	0.001	37[b]	37
	0.002	77	74
	0.004	120	150
	0.008	260	300
Smith Pond	0.1	6	7
	0.2	10	15
	0.5	29[b]	29
	1	57	58
	2	110	120
	4	170	240
	8	330	480
Pure culture fecal coliform organism (+ + − −)	0.5×10^{-6}	7	8
	1.0×10^{-6}	12	17
	2.5×10^{-6}	33[b]	33
	5.0×10^{-6}	82	66
	10×10^{-6}	130	130
	20×10^{-6}	230	260
	40×10^{-6}	450	520

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[a] Fecal coliform bacterial counts are based on the average value of 3 replicates.

[b] Optimum density used for base line calculated colony counts.

tionship exists between sample volume and fecal coliform count over a varying part of the range of sample volumes used. This proportional relationship becomes nonlinear when the total number of fecal coliform colonies, plus any non-fecal coliform bacteria colonies present exceed approximately 80 per membrane filter. Sample quantities that will yield counts between 20 to 60 result in greater accuracy of density determination. The lower limit of 20 fecal coliform colonies is suggested to reduce statistical errors caused by random sampling variations. Colony counts above 60 per membrane become inaccurate because of crowding, confluency, and competition for nutrients by the numerous colonies retained on a filter. The same limitations as to sample volumes are applicable also to other media and methods devised for the membrane filter technique.

EVALUATION OF METHODS*

The modern interpretation of the presence of the coliform group as a pollution indicator depends on the objectives of the pollution investigation. In some studies, the total coliform group will be the indicator of choice, whereas in another investigation with a different objective a portion of the group or even a single species is utilized. The object of this section is to develop criteria for interpreting the significance of the coliform group or some of its subdivisions in the investigation of polluted waters.

The scientific basis for the use of the coliform group as a pollution indicator has already been reviewed. The two extreme views of Smith(*3*) and Eijkman(*37*) as to the significance of the coliform group as an indicator of pollution have carried over to the present time. The 11th edition of *Standard Methods*(*19*) follows the Smith theory in that it does not recognize the native habitat of the coliform group as having any significance in the interpretation of pollution. It defines this group as consisting of aerobic and facultatively anaerobic, gram-negative, rod-shaped bacteria, which produce gas from lactose in 48 hours or less when incubated at 35°C. Most British bacteriologists accept an intermediate view and recognize that coliform bacteria incubated at 44°C in a modified elevated-temperature test represent more dangerous pollution than the group able to produce gas from lactose at only 37°C incubation. Some European cities use the highly restrictive procedure of Eijkman and incubate the coliform bacteria for the fermentation of glucose or lactose at 46°C.

A detailed presentation of the methods and definitions used in the quantitative detection of coliform organisms is not intended here. Bacterial specifications and methodology are mentioned only to the extent they are essential to the discussion.

The first problem facing the investigator of water pollution is the choice of procedure for the quantitative determination of indicator microorganisms in the water. The second problem is the determination of the probable native habitat of the microorganisms insofar as it might influence the proposed water uses. From these two considerations, the choice of the pollution indicator and the detection methods to be used are determined. The coliform group or some portion of it is used to indicate the sanitary quality of waters. Beyond this point, judgment, experience, and a complete knowledge of the intended water uses are required.

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Origins of Coliform Organisms

Competent investigators have repeatedly demonstrated that members of the coliform group of bacteria are found in the feces of warm-blooded animals, in the guts of cold-blooded animals, in soils, and on many plants. Warm-blooded-animal feces from humans, animals, or birds may at any time contain disease-producing microbes. These may be enteric bacterial pathogens, other disease-producing microorganisms such as some members of the *Mycobacterium* or *Brucella* genera, viruses, or intestinal parasites. It is reasonable to assume that cold-blooded animal feces are quantitatively insignificant as a source of pollution and, therefore, need not be considered. Coliform bacteria from plants or soils that have been recently exposed to fecal pollution have the same significance as those from feces; on the other hand, the coliform-bacteria contribution from soils or plants that have not been exposed recently to fecal contamination has less public health significance. All these sources are usually present in the surface water environment and contribute to the bacterial flora in it.

The objective of using the coliform group as an indicator of the sanitary quality of water is to determine that the water is free from pathogenic entities. To exclude the possibility of pathogens contributed from feces, freedom from coliform organisms must be demonstrated. Because of the number of sources contributing coliform bacteria to surface water, the more information that can be obtained as to these sources, the greater the value of the organisms as indicators of pollution. The following criteria are suggested to resolve some of the difficulties in the interpretation of data with respect to the presence of coliform bacteria in various types of waters.

Suggested Criteria

It has been repeatedly demonstrated by standard bacteriologic procedures that adequate treatment will produce potable waters in which no coliform bacteria can be detected. Kabler and Clark *(69)* have emphasized that the presence of any member of the coliform group in a treated potable water is not acceptable, regardless of its source. The presence of coliform bacteria in the potable water indicates improper practices. These may be poor plant design, improper operating procedures, poor sanitary practices or housekeeping in the plant operation, or faults or aftergrowths in the distribution system. Adequate treatment and operation will remove all coliform bacteria from any of the sources enumerated. Therefore, after proving the presence of coliform bacteria, the investigator need not be concerned with further identification of their origin. The procedures to use for the detection of the coliform group are the Presumptive Test, the Confirmed Test, and, if necessary, the Completed Test described in *Standard Methods*(*19*). Other bacteriologic tests for the origin of the coliform bacteria may be used as an aid in operation of the plant, but the results of such tests should not be used to evaluate the sanitary or the public health significance of the organism.

Standard Tests

The Presumptive Test is a screening procedure in which a negative reaction excludes the presence of the coliform group and a positive reaction indicates a probability of its presence. In the Confirmed Test, a negative reaction excludes the presence of the coliform group in samples that were positive by the Presumptive Test, while the positive reaction gives more reliable evidence of the presence of the coliform group. In the Completed Test, a positive reaction proves the presence of members of the coliform group, and the negative reaction excludes their presence in samples that were positive in the Confirmed Test. The Completed Test is designed to prove the presence or the absence of the coliform group that may have public health significance, and represents the reference test for this group of coliform bacteria. It does not give any information concerning their environmental source or native habitat.

Source Differentiation

The objective of the bacteriologic examination of an untreated surface water is usually to estimate the hazard due to fecal pollution or to determine the water source that has the least fecal pollution. This examination should be designed to yield the maximum information concerning environmental sources from which various types of coliform bacteria present in the water are derived. If we accept the hypothesis that the coliform bacteria of fecal origin represent greater danger to health than those native to other environments, separation of the fecal and non-fecal groups is justified. Thus, methods must be modified to differentiate between the portion of the coliform population deriving from fecal sources and those from other environments. For this type of pollution study, the elevated-temperature confirmatory test is recommended in addition to the routine test procedures to permit the determination of the total. the fecal, and the non-fecal coliform bacteria density (the last by calculation) and the application of selective interpretation to the data.

The elevated-temperature test was designed to discriminate between the coliform bacteria strains derived from the fecal environment of warm-blooded animals and those derived from other environments. Production of gas from lactose in EC medium (composed of tryptone, lactose, bile salts, mono- and dipotassium phosphates and sodium chloride) within 24 hours at an incubation temperature of 44.5°C (± 0.5) is interpreted to indicate fecal origin. Failure to produce gas, with or without growth, indicates that the coliform group did not recently come from the fecal environment (within a year).

Procedures for the elevated-temperature test have been described by Clark et al. (*63*) and Geldreich et al. (*70*). The examination of an untreated surface water, according to these investigators, consists of the official Presumptive Test for coliform bacteria followed by a dual confirmation of each positive tube, in which one tube is used for the official Confirmed Test and a second tube of EC medium is used for the elevated-temperature test. The production of gas, described above, is evidence of the fecal source of the coliform strain, whereas the absence of gas with the elevated-temperature test and the positive Confirmed Test in the corresponding tube indicates a Confirmed Test for the non-fecal coliform strain.

The Completed Test need not be applied to a tube that is positive by the elevated-temperature test, because complete correlation exists between the positive reaction in both procedures. Coliform strains not derived from feces, however, are negative in the elevated-temperature test and positive in the Completed Test procedure.

Test Precision

Precedents for this interpretation were established in legal hearings at Sioux City, Iowa (*71*) and at St. Joseph, Mo. (*72*). The scientific basis for the interpretation of the elevated-temperature test has been established by Geldreich and his associates (*26, 27*). They report 96 percent positive reactions from 8,747 coliform strains isolated from feces of warm-blooded animals, and more than 91 percent negative reactions from the coliform strains isolated from soils collected in 26 states. All the soils sampled reportedly were remote from any known sources of recent fecal contamination.

Superior Test Procedure

The elevated-temperature test, as previously indicated, was correlated with samples of fresh warm-blooded-animal feces and with samples of soils that were believed to be remote from fecal contamination. All coliform strains isolated from feces were assumed to be fecal strains, and all strains present in the soil samples were assumed to be of non-fecal origin. Results of the various individual tests and the IMViC group tests were determined in parallel with the elevated-temperature test and correlations were made with various procedures. These data indicate the elevated-temperature test to be superior to all other procedures with the additional advantages of simplicity and a testing time of only 24 hours.

As a result of the application of the various methodologies and the information obtained as to the scientific basis for their acceptance, the interpretation of coliform data is summarized as follows:

Potable-water samples that have received any degree of treatment should be examined by means of the official bacteriologic coliform bacteria tests, including the Presumptive Test, the Confirmed Test, and, if positive, by the Completed Test. In treated waters, the separation of the various members of the coliform group by further tests according to their probable environmental origin is not required from a public health standpoint.

Untreated surface waters should be examined by not only the usual bacteriologic coliform bacteria tests used for potable waters, but also the elevated temperature tests to separate the coliform groups as to those of fecal origin and those of other origin. An increasing fecal coliform density is correlated with increasing fecal contamination.

Summation

The intended water use should determine the technical procedures used in the bacteriologic examination of the waters. Criteria are suggested for interpreting the significance of the various members of the coliform group in the investigation of water pollution. The scientific basis for the use of the coliform groups as pollution indicators is reviewed and the various methods used in interpreting coliform data are discussed.

CHAPTER III. WARM-BLOODED ANIMALS*

The coliform bacteria have long been used as an indicator of human fecal pollution in sanitary bacteriology. Techniques, however, for the separation of the members of the coliform groups into those of fecal and non-fecal origin were controversial. The literature is replete with many suggested biochemical tests involving peptones, sugars, alcohols, growth at elevated temperatures, etc., which were reported to be useful for the detection of fecal coliforms. Among these, the indole, the methyl red (MR), the Voges-Proskauer (VP), and the citrate group of tests have been the most frequently used. In 1936 Parr(*73*) suggested the mnemonic IMViC to describe the order in which the biochemical reactions of these tests would be reported. Further biochemical tests appear to be necessary for such differentiation. One such procedure, i.e., fermentation at elevated temperature, has been applied to a number of media(*50, 60, 62*) and temperature(*53, 61, 74*) modifications. The purpose has been to detect accurately, rapidly, and conveniently, and to enumerate the fecal coliforms in contrast to the non-fecal coliforms in potable waters, stream samples, sewage, and food products.

In the investigation reported here, fecal coliforms from the feces of humans, cows, pigs, sheep, chickens, turkeys, and ducks were compared as to classification and distribution. The membrane filter technique was used to separate strains of each coliform IMViC type as they were isolated from fecal samples. This procedure was selected as the best method to get a representative enumeration of the types and their percentage distribution without the problems of overgrowth by more rapidly growing types. All fecal samples were examined within 4 hours of passage.

METHODS

The procedure we used consisted of filtering serial dilutions from a 1-gram sample through membrane filters with subsequent growth on M-Endo MF medium for 18 to 20 hours at 35°C. Between 50 and 100 isolated coliform colonies were transferred to phenol red lactose broth for evidence of gas production, followed by confirmation in brilliant-green lactose bile broth. The coliforms present in the positive brilliant-green lactose bile broth tubes were streaked on EMB agar to ensure their cultural purity. From well-isolated colonies, inoculates to nutrient agar slants were made. The coliform strains were then simultaneously inoculated into BALB and EC broth, and the necessary biochemical tests were made for identification and classification by IMViC types. The IMViC procedures used are described in *Standard Methods*(*19*).

The technical methods for the elevated temperature procedures

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* This material has been published previously in *Journal Water Pollution Control Federation* (Reference 26).

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were those recommended by Clark et al.(*63*) and Geldreich et al.(*70*). The media were prepared and sterilized as recommended and the pH determined on each sterile lot. Inoculated tubes were immediately placed in a constant-temperature water bath with the water height above the level of the medium inside the tubes. The temperature of the water bath was maintained at 43°C (± 0.5) for the BALB tubes and 44.5°C (± 0.3) for the EC tubes. The inoculated EC tubes were incubated for 24 hours, then examined for any gas production. The same procedure was used for the BALB tubes after an incubation of 48 hours.

In addition to the qualitative tests, a five-tube test in three usable decimal dilutions by the confirmed and completed coliform MPN, and a fecal coliform MPN were performed on each fecal sample. The coliform tests are described in *Standard Methods*(*19*). The fecal coliform MPN consisted of confirmation of all presumptive positive lactose tubes in EC medium as described above.

RESULTS

During this study, 8,747 strains of coliform isolates were examined. This represented 50 to 200 coliform strains from each of 103 fecal samples. There were 43 samples of feces from humans, 35 of which were adults (including 10 instituional residents), and 8, children less than 5 years old. Eleven coliform types were isolated from the samples from humans (Table 11). Six predominating types (+ + − −, − − + +, + + + −, − + − −, − + − +, and + + − +) representing 99.1 percent of the coliform population (4,512 strains) in human feces, plus five "occasional" types were recovered. The livestock portion of 32 samples consisted of 10 from sheep, 11 from cows, and 11 from hogs. In this group only six coliform types were isolated. The three predominating types (+ + − −, + + + −, and + + − +) represented 99.2 percent of the 2,339 coliform strains in feces of livestock. The 28 poultry samples were from 10 turkeys, 10 chickens, and 8 ducks. Only one (+ + − −) of the six predominating types was isolated. This one type represented 97.9 percent of the total 1,896 strains examined. No single type in the "occasional" group constituted more than 1 percent of the coliform content in any sample regardless of origin: human, livestock, or poultry.

Three samples of human feces showed several interesting deviations in their percent IMViC type distribution and percent of EC and BALB positives (Table 12). Adult A initially had 96.9 percent − − + + type, which was only 0.6 percent EC positive and 1.2 percent BALB positive. After 13 months, the percent of that type remained essentially the same, 98 percent. All strains now, however, were 100 percent EC positive and 78 percent BALB positive. Eleven months later the type had changed to 100 percent + + − −, and they were 100 percent positive in both EC and BALB. Adult B retained the same type (+ + − −) at the 100 percent level, but the EC and BALB positives changed from 19 and 5.5 percent, respectively, in June 1957,

TABLE 11. OCCURRENCE OF COLIFORM TYPES IN SAMPLES OF ANIMAL FECES

Coliform type	43 human samples		32 livestock samples		28 poultry samples		Summary	
	No. strains examined	Percent occurrence	No. strains examined	Percent occurrence	No. strains examined	Percent occurrence	No. strains examined	Percent occurrence
+ + − −	3,932	87.2	2,237	95.6	1,857	97.9	8,026	91.8
− − + +	245	5.4	0	a	1	0.1	246	2.8
+ + + −	106	2.4	59	2.5	0	a	165	1.9
− + − −	99	2.2	14	0.6	20	1.1	133	1.5
− + − +	50	1.1	1	a	5	0.3	56	0.6
+ + − +	35	0.8	27	1.2	11	0.6	73	0.8
− + + +	21	0.5	0	a	0	a	21	0.2
+ + + +	6	0.1	0	a	0	a	6	0.1
+ − + +	14	0.3	0	a	0	a	14	0.2
+ − − −	2	a	0	a	2	a	4	0.1
− − − +	2	a	0	a	0	a	2	a
− + + −	0	a	1	a	0	a	1	a
Total	4,512	—	2,339	—	1,896	—	8,747	—
EC Positive	4,349	96.4	2,309	98.7	1,765	93.0	8,423	96.3
BALB Positive	4,274	94.7	2,307	98.6	1,755	92.5	8,336	95.3

[a] Insufficient number of cultures examined.

TABLE 12. SHIFTING DISTRIBUTION PATTERNS FOR THE COLIFORM POPULATION (IN PERCENT) IN THE FECES OF THREE HUMANS

Coliform type	Adult A			Adult B			Adult C		
	Sept.[a] 1957	Oct.[b] 1959	Sept.[b] 1960	June[c] 1957	July[a] 1957	Nov.[b] 1958	July[c] 1957	Oct.[b] 1958	Jan.[b] 1961
+ + — —	—	2	100	99.5	100	100	27.5	2	10
— — + +	96.9	98	—	—	—	—	1.0	84	—
— + — —	—	—	—	0.5	—	—	—	—	—
+ + + —	—	—	—	—	—	—	70.0	—	—
— + — +	—	—	—	—	—	—	0.5	—	—
+ + — +	—	—	—	—	—	—	0.5	12	90
— + + +	2.5	—	—	—	—	—	—	1	—
+ + + +	0.6	—	—	—	—	—	0.5	—	—
— — — +	—	—	—	—	—	—	—	1	—
EC positive	0.6	100	100	19.0	28.1	100	98.0	60	100
BALB positive	1.2	78	100	5.5	33.1	100	98.0	14	100

[a] 199 strains examined.
[b] 100 strains examined.
[c] 200 strains examined.

to 100 percent positive for both tests by November 1958. Adult C changed in 41 months from 70 percent + + + − through 85 percent − − + +, to 90 percent + + − +. At the same time, EC and BALB positives decreased from 98 percent for both to 60 percent EC and 14 percent BALB, and then increased to 100 percent for both media. All of the other 100 fecal samples were predominantly composed of *E. coli* type strains.

Many attempts have been made to find a satisfactory differentiation of fecal coliforms. Table 13 lists previously suggested criteria by which the strains were differentiated in this study. The livestock and poultry samples are combined as non-human coliform strains. By this analysis, the EC method detected 96.4 percent of all human-derived coliform strains and 96.2 percent of all non-human fecal coliform bacteria. The BALB test detected 94.7 percent human strains and 95.9 percent non-human coliform organisms. The methyl red test yielded the highest detection: 99.9 percent of all strains from the livestock and poultry. This test was slightly lower, however, in detecting the human coliforms (94.2 percent) than either the EC or BALB elevated temperature tests. There was a 4.5 percent difference between these 10 methods for detecting fecal coliforms when applied to the non-human group of warm-blooded animals, while a 9.2 percent variation was seen for the human strains.

The estimated per capita number of coliforms discharged daily is listed in Table 14 for each of the warm-blooded species represented in this survey. These figures are based on the average wet weight of a 24-hour fecal discharge and the median coliform density per gram of wet weight as determined from the completed MPN test. Such calculations prove of value for stream pollution studies in estimating the quantity of pollution contributed by farm animals located in a watershed or in a surface drainage area.

DISCUSSION

The frequency of occurrence for + + − − and − − + + types in the warm-blooded animals is similar to that generally reported in the literature. Samples from 10 adult institutional residents who lived in a controlled environment, ate the same food, and worked on a dairy farm, demonstrated the same coliform distribution as the other 33 human samples from varied environments. Human fecal samples contained the greatest variety of coliform types; in this case, 11 of the possible 16 coliform types. The feces of the other animals were almost exclusively + + − −, with only five other types occasionally appearing. Growth at elevated temperature did not appear to be specific for any one type or group of types. This would indicate that in warm-blooded animals coliforms with the ability to produce gas in lactose at 44°C comprise 96 percent or more of the strains isolated.

Of the 103 samples examined, only three human samples showed several interesting deviations; these occurred either in their coliform type composition, their elevated temperature results, or both. The

TABLE 13. DIFFERENTIATION OF COLIFORM STRAINS BY SUGGESTED CRITERIA

Criteria	Literature reference No.	Human-derived coliform strains[a]				Non-human-derived coliform strains[b]			
		Fecal		Non-fecal		Fecal		Non-fecal	
		No.	%	No.	%	No.	%	No.	%
+ + − −	49	3,932	87.2	580	12.8	4,094	96.7	141	3.3
+ + − − and − + − −	19	4,031	89.3	481	10.7	4,128	97.5	107	2.5
BALB+, Indole+	63	4,058	89.9	454	10.1	4,040	95.4	195	4.6
EC+, Indole+	53	4,060	90.0	452	10.0	4,043	95.5	192	4.5
Indole+	57	4,095	90.8	417	9.2	4,193	99.0	42	1.0
MR+, VP−	75	4,116	91.2	396	8.8	4,172	98.5	63	1.5
Citrate−	45	4,139	91.7	373	8.3	4,190	98.9	45	1.1
MR+	17	4,249	94.2	263	5.8	4,232	99.9	3	0.1
BALB+	50	4,274	94.7	238	5.3	4,062	95.9	173	4.1
EC+	62	4,349	96.4	163	3.6	4,074	96.2	161	3.8

[a] Percentages are based on 4,512 cultures examined.

[b] Percentages are based on 4,235 cultures examined.

TABLE 14. ESTIMATED DAILY PER CAPITA DISCHARGE IN ANIMAL FECES

Item	Human	Cow	Hog	Sheep	Ducks	Turkeys	Chickens
Moisture content, %	77.0	83.3	66.7	74.4	61.0(*76*)[a]	62.0(*76*)[a]	71.6
Average weight of 24-hr fecal discharge, wet weight in grams	150(*77*)[a]	23,600(*78*)[a]	2,700(*78*)[a]	1,130(*78*)[a]	336(*79*)[a]	448(*79*)[a]	182(*80*)[a]
Coliforms per gram, millions	13	0.23	3.3	16	33	0.29	1.3
Total coliforms discharged per day, millions	1,950	5,428	8,910	18,080	11,088	130	237

[a] Parenthetical numbers are literature references.

variability in persistence of coliform types in human feces has been observed by Parr(*81*) and more recently by Sears, Browles and Vchiyama(*82*). The additional shift in EC and BALB positives could be one possible reason for the occasional fecal sample that does not yield the expected positive elevated temperature results.

Information in Table 13 shows that either of the elevated temperature tests for the differentiation of fecal coliforms in human feces is superior to any other single test in this study. This superiority was not evident for other warm-blooded-animal feces, probably because the IMViC type + + − − was excessively predominant. In addition to its efficiency in designating fecal types, the elevated temperature test requires less material and labor and shorter time for completion.

The high correlation of elevated temperature positive reactions and fecal source indicates that EC and BALB positive coliform strains are probably of fecal origin even when recovered from water or wastes of undetermined pollution content. The data presented strongly support this interpretation. The recommended procedure for a sanitary examination includes an initial presumptive test in lactose broth or lauryl tryptose broth. A preliminary enrichment in a non-selective medium is essential because the elevated temperature tests are most efficient when coliform bacteria are used in the logarithmic growth phase. The positive presumptive tubes are confirmed in brilliant-green lactose bile broth at 35°C for total coliform density and in EC medium at 45°C for fecal coliform density. This procedure gives maximum information with total coliforms, fecal coliforms, and non-fecal coliforms (by calculation). Because the preponderance of coliform strains in the other warm-blooded animals was + + − −, very little difference was noted in the efficiency of differentiation between the suggested fecal coliform tests. Not only are both elevated temperature procedures as efficient for fecal coliform detection in livestock and poultry samples and better with human samples, but both require less time and work than a biochemical series of the four IMViC tests.

This suggests that the IMViC biochemical series is not the best answer in defining the fecal coliform group in water and wastes. The elevated temperature test used as a confirmatory procedure is suggested as a possible solution.

Prior research has indicated the need for an enrichment period in lactose or LST broth to get optimum recovery of all levels of fecal coliforms that might be present in a water sample. In any event, further consideration should be given to the development of other measurements that show better correlation with fecal or non-fecal origins of the coliform group.

SUMMATION

In this investigation, we examined the reactions of 4,512 human, 2,339 livestock, and 1,896 poultry coliform strains isolated from 43

GPO 828—355—3

human, 32 livestock, and 28 poultry fecal samples. The EC procedure showed a 96.3 percent positive correlation and the BALB procedure a 95.3 percent correlation. These findings suggest that the presence of EC or BALB positive coliform strains in water or wastes indicates relatively recent fecal pollution. An occasional shift in percent positive correlation of the elevated temperature test with three human samples during a period of 1 to 3 years was noted. The biochemical tests comprising the IMViC typing classification are cumbersome and do not present as close a correlation with fecal origins of the coliform group as other procedures. In view of the excellent reliability of either the EC or BALB test for fecal coliform bacteria and the saving in time and effort, the elevated temperature procedure seems to be the best method.

CHAPTER IV. FRESH-WATER FISH*

In recent years, considerable interest in studying the occurrence and distribution of the various bacterial pollution indicators in water and the associated environment has been apparent. Reports of studies on potential natural pollution of water from warm-blooded animals, soils, vegetation, and insects have appeared in the literature. It has been suggested repeatedly that the bacterial flora of fish might reflect the bacteriological conditions of the water and thus be a potential indicator of pollution. This thesis assumes that coliforms, fecal coliforms, and fecal streptococci are not normal inhabitants of the gut of fish and have a short, unaltered survival in their intestinal tract. Little quantitative data can be found in the literature, however, on either the pollution that might be contributed by fish, or the degree of correlation between water quality and the number of bacterial pollution indicators in the fish intestine.

Organisms of the coliform and streptococcus groups have been isolated from the intestinal tract of various species of fresh-water fish caught in relatively clean to moderately polluted waters in India (Venkataraman and Sreenivasan, *83*), Norway (Thjotta and Somme, *84*), Canada (Amyot, *85*; Margolis, *86*; Evelyn and McDermott, *87*), and the United States (Johnson, *88*). Margolis(*86*) also reported that the bacterial flora of fresh-water fish depends solely upon the fish's recent intake of food and the degree of contamination in the food and water. Gibbons(*89, 90*) reported similar results for fish caught from the marine environment, and both authors concluded that coliform bacteria are not usually associated with the normal intestinal flora of fish.

Although numerous microbiological studies have been made on fish intestinal material, much of the data are qualitative and are based on technical procedures with limited sensitivity and selectivity. Insufficient data in the literature on quantitative recovery of coliforms, fecal coliforms, and fecal streptococci that could be applied to water pollution studies prompted this investigation. The research project was developed to include the following areas: (1) Quantities of coliforms, fecal coliforms, and fecal streptococci in the intestinal contents of fish; (2) possible correlation of these pollution indicators in fish with those in the water environment; (3) survival and multiplication of fecal coliforms and streptococci in intestinal material; (4) determination of any density differences and possible type differences of pollution indicators in the upper and lower intestine; (5) occurrence and percentage distribution of coliforms and streptococcus strains from the gut of a wide variety of fish; (6) the effect of ingestion of specific tracer organisms and their retention in the fish intestine; and (7) contribution of fish to pollution of a potable water.

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* Material in this chapter was published previously in *Applied Microbiology* (Reference 151).

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MATERIALS AND METHODS

Bacteriological examinations were conducted as soon as possible after the fish were caught, except for fish caught in areas of Canada; Pymatuning Lake, Pennsylvania; and Dale Hollow, Tennessee, where the intestinal tracts were immediately removed aseptically and frozen for prompt shipment to the laboratory. Fish caught in the Ohio and Little Miami Rivers, in several small Ohio lakes, and those from experimental fish tanks were placed in plastic bags and examined immediately on return to the laboratory.

Most of the fish were taken with fish traps and large river nets set in the streams for 1 to 2 weeks in summer; a few were obtained by seining with a gill net, and the remainder were line caught. Fish used in the 250- and 500-gallon tank experiments were taken with dip nets.

To determine the flora of the intestinal tract, the entire gut from stomach to anus was removed and the contents stripped out with sterile forceps. In many of the samples that contained large amounts of fecal material, a minimum amount of 0.5 gram (wet weight) was obtained from both the upper and lower intestinal tract for use in split-sample studies. After they were weighed, the samples were transferred to appropriate amounts of sterile water to make a 1:100 dilution. The material was homogenized in the dilution water by shaking with glass beads.

Fecal suspensions were examined for coliform bacteria by the "Confirmed" and "Completed" multiple-tube most probable number (MPN) procedures as described in *Standard Methods*(*19*). The fecal coliform test described by Geldreich, et al.(*26*) consisted of confirmation of all positive presumptive tubes in EC broth at 44.5°C (± 0.5). MPN for the fecal streptococcus group was determined by the "Tentative Test"(*19*) with azide dextrose (AD) presumptive broth followed by confirmation of all positive tubes in ethyl violet azide (EVA) medium. All confirmed positive EVA tubes were verified by microscopic examination to be gram positive cocci. Pour plates of KF streptococcus agar(*91*) were used for the isolation of colonies to be studied in subsequent species or group classification for the streptococcal types present. All quantitative data were reported as number of organisms per gram of sample.

Coliform strains used in the study of biochemical reactions were obtained through a primary isolation of colonies growing on the membrane filter. At least 20 to 50 isolated colonies with typical sheen were transferred to phenol red lactose broth for fermentation tests and verified in brilliant green lactose broth. Streaking on eosin methylene blue (EMB) agar insured cultural purity. All purified strains were then inoculated into EC broth at 44.5°C and introduced into the media necessary for identification and classification by IMViC types(*19*).

Possible survival and multiplication of a fecal coliform (+ + − − IMViC type) and *Streptococcus fecalis* var. *liquifaciens* in the intestinal material of fish were also investigated. To demonstrate growth within this material, intestinal contents of six bluegills and eight carp were prepared as a 1-percent suspension with triple distilled water. These "media" were then chemically sterilized with ethylene oxide and heated to 60°C for 6 hours to drive off residual ethylene oxide. Flasks of these "media" were individually inoculated with pure cultures of the + + − − fecal coliform and *S. fecalis* var. *liquifaciens* and incubated at either 10°, 20°, or 35°C. Plate counts on brain heart infusion agar were made initially and after 24, 48, and 72 hours to obtain bacterial densities.

RESULTS AND DISCUSSION

Percentile distributions for the MPN values of the five-tube coliform "Completed Test" and for the fecal coliform and fecal streptococcus group tests on various species of fish and their river water environments are summarized in Table 15. Coliforms, fecal coliforms, and fecal streptococci were found in many of the 78 specimens of fish examined, including bluegill (*Lepomis macrochirus macrochirus*), bass (*Micropterus salmoides*), white crappie (*Pomoxis annularis*), gizzard shad (*Dorosoma cepedianum*), white sucker (*Catostomus commersonii commersonii*), northern redhorse (*Moxostoma aureolum*), carp (*Cyprinus carpio*), and channel catfish (*Ictalurus lacustris punctatus*) caught in moderately polluted sections of the Little Miami River. Fecal coliform densities were lowest in bluegills; in 75% of the samples examined, the value was below the minimum level that could be detected in the amount of fecal material available. The 330 coliform and 220 fecal streptococci values at the 75 percentile level reflect the low densities of these indicators also in bluegill fecal material. Catfish contained the largest bacterial concentration, with upper quartile values of 7,900,000 coliforms, 1,090,000 fecal coliforms, and 240,000 fecal streptococci per gram of feces. All fish used in these experiments were caught during July, August, and September when the water temperature was between 13° and 18°C.

Examination of water sampled at the various fish-trap locations indicated lower densities for the pollution indicators in river water than in the fish. Upper quartile values for total coliforms, fecal coliforms, and fecal streptococci were 2,200, 370, and 100 per 100 milliliters, respectively. These data suggest that the major source of the pollution indicators in fish from the Little Miami River was probably related to the intake and degree of contamination of food ingested. Margolis(*86*) reported the same observation in a study of the effect of fasting on bullheads and speckled trout in tank experiments. Our tank experiments with carp and bluegill indicated a higher recovery for the pollution indicators in fish guts when the water temperatures ranged from 16° to 21°C than when they ranged from 1° to 10°C. In the latter case, fish were not actively feeding and intestinal tracts were mostly empty.

Another possible reason for the higher counts in fish than in their water environment could be a result of bacterial multiplication within

TABLE 15. PERCENTILE DISTRIBUTION OF BACTERIA BY QUARTILE OF MPN VALUES PER GRAM FISH INTESTINAL MATERIAL[a]

Fish species	No. of specimens	Test								
		Completed coliform			Fecal coliform			Fecal streptococci		
		25%	50%	75%	25%	50%	75%	25%	50%	75%
Sunfish										
Bluegill	6	<20	20	330	<20	<20	<20	<20	<20	220
Bass	13	81	490	221,000	<20	330	23,000	23	60	79,000
White crappie	9	<20	3,300	490,000	<20	330	7,900	49	240	23,000
Herring										
Gizzard shad	11	33	490	79,000	8	70	46,000	13	230	10,900
Suckers										
White sucker	6	490	630	7,000	50	70	490	79	79	940,000
Northern redhorse	6	13,000	17,200	172,000	2,300	4,600	13,000	42,600	130,000	790,000
Minnows										
Carp	5	17,200	221,000	278,000	1,300	7,900	7,900	3,300	3,300	6,300
Catfish										
Channel catfish	22	84,000	221,000	7,900,000	3,300	49,000	1,090,000	3,300	130,000	240,000
Little Miami River[b]	13	141	1,700	2,200	55	300	370	22	40	100

[a] All fish were caught in the Little Miami River during July, August, and September.

[b] Densities per 100 ml of water.

the fish gut. Persistence of a fecal coliform, a fecal streptococcus, *Salmonella typhosa*, *Salmonella typhimurium*, and *Shigella flexneri* in previously sterilized fish fecal material is demonstrated in Figures 2 and 3. Subjecting this material to ethylene oxide sterilization does eliminate any potential composite effect of interaction and interrelationships between the original microbial flora and the persistence of experimental organisms. The influence, however, of such environmental factors as pH of fecal material, chemical toxicity of waste products, and temperature variations can be observed in growth studies of sterilized fecal discharges.

The data presented in Figure 2 were obtained in studies of fecal material from carp caught in several locations, whereas Figure 3 shows similar data for bluegill. The curves are based on average values obtained from 3 to 11 replicate experiments for each bacterial strain and fish species. These data indicate a progressive die-away for the fecal coliform and three pathogenic strains of bacteria at 10°C, with only a slight increase of approximately two generations in 72 hours for fecal streptococci in fecal material from both carp and bluegill. Since body temperatures of fish are essentially the same as the temperature of the water they inhabit, data from the 10°C experiments suggest that no significant multiplication of the pollution organisms occurs in the gut of fish over a 3-day period during winter and early spring.

When the experiments with both species were repeated at 20°C (summer water temperature), a pronounced increase in growth of fecal streptococci occurred within 48 hours. The bluegill samples had, in addition, a similar increase in bacterial density for the fecal coliform, *S. typhimurium*, and *Sh. flexneri*. With 72-hour incubation, some growth occurred for the fecal coliform and *S. typhimurium* in carp fecal material, but not of the magnitude found in bluegills. In fecal material from bluegills, *Sh. flexneri* significantly increased with 48-hour incubation, but was completely killed in carp material for the same time interval. These bacterial growth differences in the two species of fish may be related to food source, waste products, and pH of the intestinal material. The pH of carp fecal material averaged 7.3 while that of the bluegill was 8.0. The magnitude of growth of these organisms in living fish could be altered by the presence of a mixed microbial population containing phages or antagonistic substances of pseudomonads. Havens and Dehler(92) found evidence that the presence of *Pseudomonas aeruginosa* in the intestinal flora of the top minnow, *Gambusia affinis*, inhibited the growth of *E. coli*. The blue-green alga, *Schizothrix calcicola*, which developed a dense "algal bloom" in one of our outdoor experimental tanks, was also demonstrated by laboratory experiments to be toxic to a fecal coliform strain within 24 hours. If fish had ingested large amounts of this alga, it is assumed a similar toxic reaction might have occurred in their intestinal tract.

While these factors might limit survival or growth of pollution indicators and pathogens in the fish intestinal tract, there is also evi-

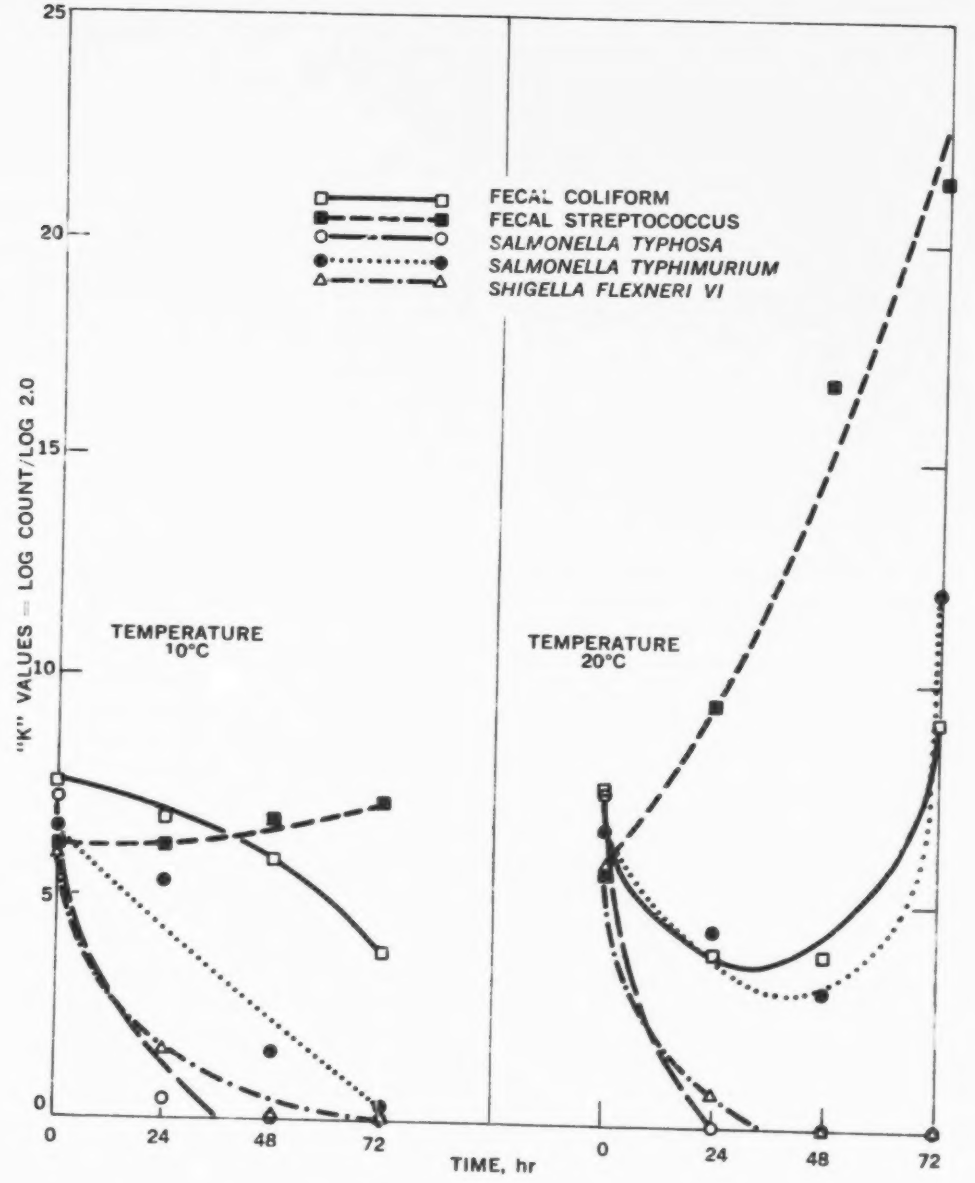

Figure 2 — Persistence of selected enteric bacteria in carp intestinal material.

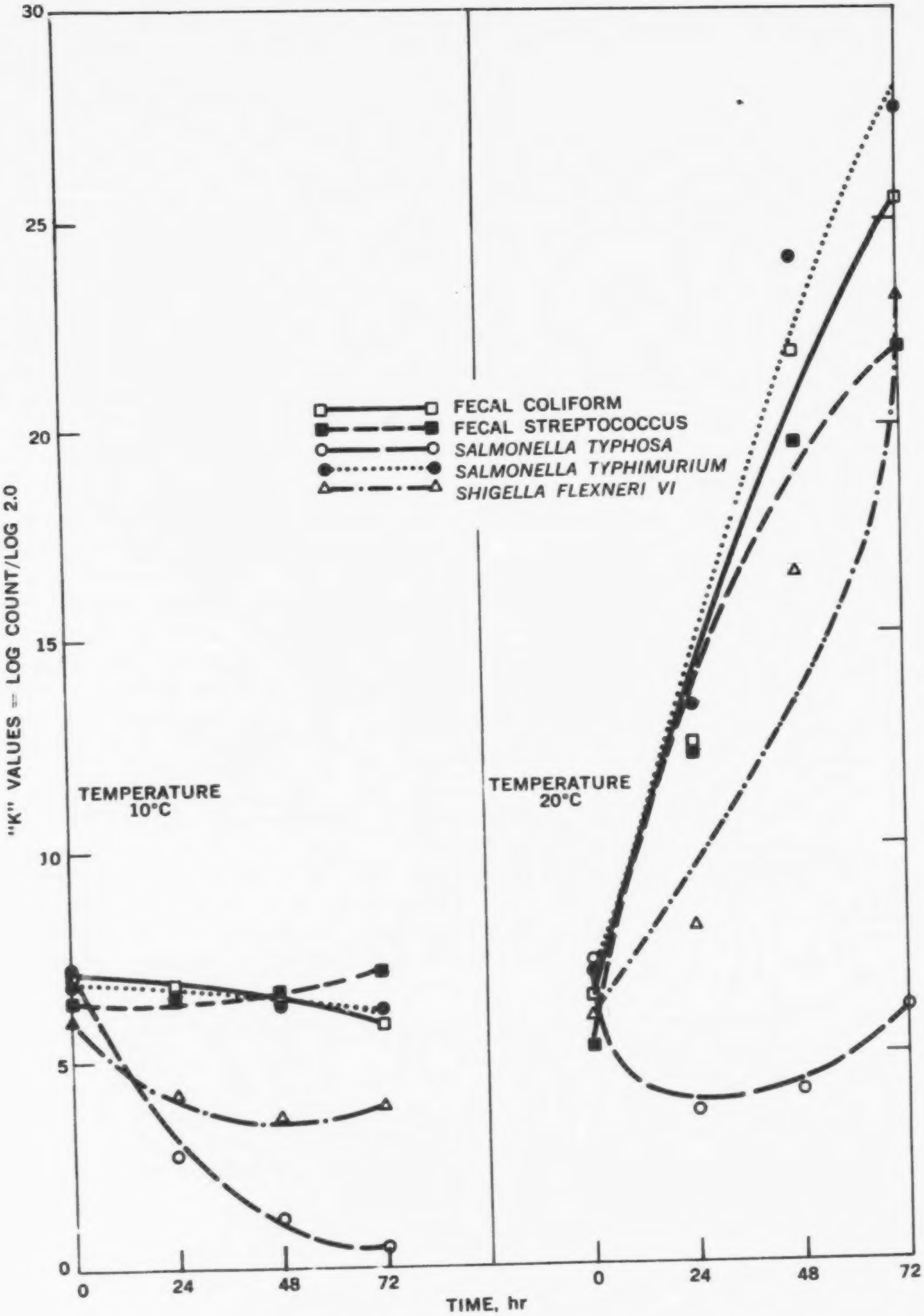

Figure 3 — Persistence of selected enteric bacteria in bluegill intestinal material.

dence that growth might occur if conditions of temperature and retention of feces in the intestine are favorable. Retention of fecal excreta is related to gut motility, and data on bacterial densities for the three pollution indicators in the upper and lower intestine (Table 16) suggest such retention. Examples of bacterial counts in six fish species indicate a definite trend toward higher densities for total coliform, fecal coliform, and fecal streptococci in material from the intestinal sections adjacent to the anus. Since fish do not have an indigenous bacterial flora (Margolis, *86*; Zobell, *93*; Potter and Baker, *94;* and Guelin, *95*) these pollution organisms must have been introduced into the fish by contaminated food and water. The resulting increases in densities in the lower gut may be related to slow gut motility which, coupled with warm summer water temperatures, would permit some bacterial multiplication prior to excretion. Note the low order of increase in densities for intestinal material from the upper and lower gut of carp in winter. No evidence of toxicity to *E. coli* or *S. fecalis* that might be associated with bile or low pH in the upper intestinal material could be found in various split samples.

The biochemical reactions of 3,877 coliform strains isolated from 132 fresh-water fish of 14 different species are reported in Table 17. These data are divided into fish groups or individual species with similar eating habits. Only the IMViC $^{-+-+}$ and $^{++--}$ types reached percentages that might be considered predominant. The $^{-+-+}$ coliform type occurred most frequently in sunfish, suckers, and carp, while $^{++--}$ type was most common to gizzard shad and catfish. Seaburg and Moyle(*96*) studied feeding habits of fresh-water fish and found that bluegills, pumpkinseeds, and crappies feed mostly on insects in the early summer. Previous studies of soil and insects (Geldreich, et al, *27*; Geldreich, Kenner and Kabler, *66*) indicated that $^{-+-+}$ coliform type occurred most frequently: 48.1 percent in soil and 30.6 percent from insects. Since sunfish feed on insects, the similarity of percentages for $^{-+-+}$ type in those fish and insects is interesting; also the percentage of fecal coliforms in sunfish (18.6%) and insects (14.9%). Data on coliform types from all fish indicate 91.4 percent of all strains were composed of five IMViC types. The remaining coliform types occurred in varying amounts of less than 5 percent.

The occurrence of fecal coliforms in fish could be a reflection of the pollution level of their water environment in addition to their feeding habits. Table 18 shows the fecal coliform percentage, based on EC positive strains, to be 30.8 percent for fish, which is essentially identical with that for water (31.0%) from the same location in the Little Miami River. Analysis of the coliform types from Little Miami River fish indicated 86.9 percent of all types were represented by $^{++--}$, $^{--++}$, $^{-+-+}$, and $^{++++}$. These same four types were represented in 79.3 percent of all coliforms isolated from fish collected at this location.

Experiments with bluegills and carp kept in 250-and 500-gallon tanks at the Newtown (Ohio) Fish Hatchery also support the thesis

TABLE 16. BACTERIAL DENSITIES (PER GRAM) IN UPPER- AND LOWER-INTESTINAL CONTENTS OF FISH

Fish species	Water temperature	Total coliform		Fecal coliform		Fecal streptococci	
		Upper	Lower	Upper	Lower	Upper	Lower
Bluegill	Summer (16° - 20°C)	26	172	< 2	< 2	< 2	< 2
River quillback		490	790	9	70	79	79
Northern redhorse		130	2,120,000	33	7,900	12	17,200
White sucker		630	33,000	79	10,900	21	7,900
Carp		17	3,300	5	490	< 2	< 2
Catfish		7,900	84,000	1,720	3,300	1,300	3,300
Carp A	Winter (1° - 10°C)	11	17	< 2	< 2	130	4
Carp B		2	< 2	< 2	< 2	23	70
Carp C		34	39	< 2	< 2	< 2	< 2
Carp D		2,210	17,700	< 2	2	< 2	33

TABLE 17. OCCURRENCE OF COLIFORM TYPES IN FECAL MATERIAL FROM FRESH-WATER FISH

IMViC type	Sunfish[a]		Gizzard shad		Sucker[b]		Carp		Catfish		Summary	
	No. of strains examined	% isolated	No. of strains examined	% isolated	No. of strains examined	% isolated	No. of strains examined	% isolated	No. of strains examined	% isolated	No. of strains examined	% isolated
+ + − −	185	18.9	92	41.4	131	34.3	73	9.1	572	38.3	1,053	27.2
− − + +	49	5.0	47	21.2	25	6.5	56	7.0	206	13.8	383	9.9
− + − −	16	1.6	2	0.9	7	1.8	13	1.6	38	2.5	76	2.0
+ + + −	0	0.0	3	1.4	3	0.8	14	1.8	7	0.5	27	0.7
− + − +	434	44.4	31	14.0	156	40.8	289	36.1	360	24.1	1,270	32.8
+ + − +	246	25.1	4	1.8	11	2.9	152	19.0	41	2.7	454	11.7
− + + +	19	1.9	6	2.7	6	1.6	66	8.3	40	2.7	137	3.5
+ + + +	24	2.5	22	9.9	36	9.4	112	14.0	187	12.5	381	9.8
+ − + +	5	0.5	2	0.9	2	0.5	13	1.6	36	2.4	58	1.5
− − − +	0	0.0	0	0.0	2	0.5	1	0.1	6	0.4	9	0.2
− + + −	0	0.0	5	2.3	1	0.3	9	1.1	2	0.1	17	0.4
− − + −	0	0.0	2	0.9	1	0.3	1	0.1	0	0.0	4	0.1
+ − + −	0	0.0	6	2.7	1	0.3	0	0.0	0	0.0	7	0.2
+ − − +	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
+ − − −	0	0.0	0	0.0	0	0.0	1	0.1	0	0.0	1	0.0
Total	978	......	222	......	382	......	800	......	1,495	......	3,877	......
EC positive	182	18.6	103	46.4	154	40.3	65	8.1	720	48.2	1,224	31.6

[a] Sunfish include bluegill, bass and white crappie.

[b] Suckers include white sucker and northern redhorse.

TABLE 18. OCCURRENCE OF COLIFORM TYPES IN FISH AND THEIR WATER ENVIRONMENT

IMViC type	Little Miami River				Fish hatchery tanks			
	Fish[a]		Water		Fish[b]		Tanks	
	Number	%	Number	%	Number	%	Number	%
+ + − −	263	27.1	40	25.8	28	2.7	1	0.5
− − + +	128	13.2	35	22.6	8	0.8	0	0.0
− + − −	24	2.5	3	1.9	0	0.0	0	0.0
+ + + −	7	0.7	2	1.3	17	1.6	0	0.0
− + − +	333	34.4	36	23.2	499	48.0	124	62.0
+ + − +	47	4.9	10	6.5	320	30.8	50	25.0
− + + +	22	2.3	7	4.5	26	2.5	1	0.5
+ + + +	118	12.2	12	7.7	129	12.4	24	12.0
+ − + +	10	1.0	8	5.2	6	0.6	0	0.0
− − − +	2	0.2	0	0.0	1	0.1	0	0.0
− + + −	7	0.7	1	0.7	5	0.5	0	0.0
− − + −	2	0.2	0	0.0	0	0.0	0	0.0
+ − + −	6	0.6	0	0.0	0	0.0	0	0.0
+ − − +	0	0.0	1	0.7	0	0.0	0	0.0
+ − − −	0	0.0	0	0.0	0	0.0	0	0.0
Total	969	......	155	......	1,039	......	200	......
EC Positive	298	30.8	48	31.0	11	1.1	6	3.0

[a] Data from 8 species of fish.
[b] Data from carp and bluegills.

that the coliform types present in fish are the same as those in their water environment. The fish food contained less than 2 fecal coliforms per gram and the tank water yielded 3.0 percent fecal coliforms among the 200 strains examined. This value was essentially the same as the 1.1 percent fecal coliforms among the 1,039 coliform strains isolated from bluegills and carp in the tanks. Three coliform types (− + − +, + + − +, and + + + +) represented 91.2 percent of the strains from carp and bluegills used in these tank experiments, whereas the same three coliform types were represented in 99.0 percent of the strains isolated from the tank water.

The results of the biochemical tests with 850 streptococci isolated from the fecal material of 55 fresh-water fish are given in Table 19. The species of fish represented included bluegill, bass, white crappie, gizzard shad, white sucker, carp sucker, northern redhorse, carp,

TABLE 19. BIOCHEMICAL REACTIONS OF 850 STREPTOCOCCI ISOLATED FROM FECAL MATERIAL OF 55 FRESH-WATER FISH[a]

	Growth at		Growth in		Reduction of					
No. of strains	10°C	45°C	1.5% NaCl	pH 9.6 broth	Methylene blue (0.1%)	Potassium tellurite 1:2,560	Final pH in 1% glucose	Hydrolysis of starch	No. of strains peptonizing milk	Arginine hydrolysis
535	+	+	+	+	+	+	4.0 — 4.7	0	119	+
16	+	+	+	+	0	+	4.1 — 4.4	0	9	+
5	0	+	+	+	0	+	4.9 — 6.5	0	0	+
43	+	+	+	0	+	+	4.1 — 4.4	0	0	+
3	0	+	+	0	+	+	4.8 — 6.4	0	0	+
48	0	+	+	0	0	+	4.8 — 6.5	0	0	+
33	+	+	+	+	0	+	4.3 — 5.2	0	0	0
29	+	+	+	+	+	+	4.3 — 4.4	0	6	0
1	+	+	+	+	+	0	4.5	0	0	0
1	+	+	0	+	+	0	4.2	0	0	0
16	+	+	0	+	+	+	4.2 — 4.4	0	0	+
32	+	+	0	+	0	+	4.2 — 4.4	0	0	+
2	0	+	0	+	0	+	4.2 — 4.4	0	0	+
22	+	+	0	+	0	+	4.4	0	0	0
1	+	+	0	+	+	+	4.4	0	0	0
1	+	+	0	0	0	+	4.3	0	0	+
1	+	+	0	0	0	0	4.3	0	0	+
14	+	0	+	+	+	+	4.2 — 4.4	0	0	+
26	+	0	0	+	±	+	4.2 — 4.5	0	0	+
21	+	+	+	+	+	+	4.2 — 4.7	+	3	+

[a] All strains catalase negative; all grow in 40% bile broth.

catfish, and northern pike. A preliminary analysis of data from different species of fish or fish of similar feeding habits did not reveal any significant differences; therefore the data are presented in a summary table.

The predominant streptococci grew at both 45° and 10°C and were found in 88.5 percent, or 752 of the total 850 strains. These included *S. fecalis* and various closely associated biotypes. Only 58 strains, or 6.8 percent, of the streptococci were capable of growth at 45° and not at 10°C. Such a grouping would include one or more of the following strains: *Streptococcus bovis*, *Streptococcus equinus*, and *Streptococcus salivarius*. Finally, only 40 strains were capable of growth in brain heart infusion broth at 10°, but not at 45°C; these included *Streptococcus lactis*, among others.

A previous study of the streptococci isolated from insects (Geldreich, Kenner, and Kabler(*66*) demonstrated that peptonization of milk appears to be a relatively common characteristic of fecal streptococci isolated from insects. In that investigation, 45.6 percent of all streptococcus strains from insects were positive. Because some fish feed on large quantities of insects, the data were analyzed for any significant percentage of litmus milk peptonization. No positive strains were found in fecal material from pike. Seaburg and Moyle (*96*) examined the stomach contents of 203 northern pike and found fish to be the major food item in all but one, which consisted only of frogs. Although gizzard shad are normally considered to feed primarily on plankton, Price(*97*) found that they may on occasion feed heavily on insects. Isolation of streptococcus strains in his study of gizzard shad showed 59.6 percent or 28 strains were positive for litmus milk peptonization, which indicated they possibly were derived from insects.

To summarize all the streptococcus data on milk peptonization in fish, 137 strains or 16.1 percent of the 850 cultures were positive. In our study of 3,158 streptococcus strains from feces of warm-blooded animals, we found a similar distribution of organisms with this characteristic, i.e., 16.9 percent or 533 positive cultures. Thus, the data presented here indicate that streptococci that peptonize litmus milk are not permanent residents of the fish gut and, when present, may be related to the intake of insects as food.

An experiment was conducted in which carp and bluegill were exposed to fecal coliform and fecal streptococcus tracer strains that were added to tank water, but not to the food. The fecal coliform strain was capable of H_2S production at 44.5°C, and the fecal streptococci peptonized litmus milk in 18 to 24 hours. These two organisms were not detected in the tank water, fish food, or fish prior to experimental dosage. The tank was dosed with saline-washed cells of 24-hour-old cultures of the two tracer organisms. Then, after various exposure periods, fish were sacrificed for evidence of ingestion and retention of these organisms in their intestinal tract.

Data in Table 20 demonstrate that tracer organisms ingested by

TABLE 20. EXPERIMENTAL EXPOSURE OF CARP AND BLUEGILL TO FECAL COLIFORMS AND FECAL STREPTOCOCCI TRACER STRAINS ADDED TO TANK WATER[a]

Tank water,[b] counts per 100 ml				Fish guts, counts per gram		
Time after dosage	pH	Fecal coliform	Fecal streptococci	Species	Fecal coliform	Fecal streptococci
1 hour	7.1	79,000	7,000,000	......	......	......
1 day	7.1	13,000	141,000	......	......	......
7 days	7.1	27	460	Carp	< 2	9
				Bluegill	490	1,720
9 days	6.9	5	130	Carp	11	4,600
				Bluegill	< 2	26
14 days	6.5	< 2	23	Carp	23	230
				Bluegill	< 2	< 2
16 days	6.8	< 2	23	Bluegill	< 2	< 2

[a] No fecal coliform or fecal streptococci detected in fish, water or food prior to dosing.

[b] Tank water temperatures ranged from 16° — 18°C.

bluegills and carp were retained in the intestinal tract for 9 to 14 days. These (and other data not included in this table) show that the contamination of the water environment must remain at high levels if continued recovery of these organisms from fish guts is to be expected. Similar conclusions for *E. coli* serotypes in trout were reported by Glantz and Krantz(*98*). They detected *E. coli* serotypes for periods of 1 to 14 days in trout that received *E. coli*-dosed food and water prior to retention studies.

Once fish have acquired pollution or pathogenic organisms, or both, in their gut, it is conceivable that they could become carriers of such bacteria to clean stream areas some distance from a polluted water environment. Results of tank experiments designed to study contamination of a potable spring water by fish exposed to high levels of coliforms and fecal streptococci are reported in Table 21. At the time of transfer of three carp and three bluegills, the tank contained approximately 250 gallons of spring water and was aerated to maintain sufficient dissolved oxygen to support the fish life. Results indicated that contamination of the potable spring water was caused by organisms associated with fish intestinal wastes and slimes from body surfaces. Similar experiments performed at tank temperatures of 1° to 5°C and 7° to 10°C gave essentially the same results.

The data presented constitute strong evidence that there is no permanent coliform or streptococcal flora in the intestinal tract of fish. The composition of the intestinal flora is related in varying degrees to the level of contamination of water and food in the environment.

TABLE 21. CONTRIBUTION OF POLLUTED FISH TO POLLUTION OF POTABLE SPRING WATER[a] IN TANK EXPERIMENTS

Tank water,[b] MPN counts per 100 ml					Fish guts, MPN counts per gram			
Time after fish added	pH	Total coliform[c]	Fecal coliform	Fecal streptococci	Species	Total coliform[c]	Fecal coliform	Fecal streptococci
2 days	7.2	49,000	< 2	49	Carp	330,000	< 2	23
					Bluegill	172	< 2	< 2
7 days	7.3	3,300	< 2	49	Carp	70,000	< 2	1,090
					Bluegill	< 20	< 20	< 20
9 days	7.2	27,800	< 2	1,410	Carp	24,000,000	< 2	175,000
					Bluegill	34,800,000	22	10,900,000
After fish were removed from tank water								
5 days	7.5	1,300	< 2	2				
12 days	7.3	141	< 2	17				

[a] Less than 2 total coliform, fecal coliform, and fecal streptococci per 100 ml spring water.
[b] Tank water temperatures ranged from 16° — 20°C.
[c] Completed test for total coliform densities.

These flora can be modified by many factors such as feeding activity, available food, and degree of water pollution. They can also be altered by antibiotic effects of ingested algae, pseudomonas species, or other bacteriological agents in the fish gut. Fish may also be carriers of pollution from warm-blooded animals for periods up to approximately 7 days, and could in this manner transfer potential pathogens to clean water areas.

CHAPTER V. VEGETATION AND INSECTS*

The coliform group and the fecal streptococcus group are composed of many species, of which some predominate in warm-blooded animal feces, and others predominate in soil and on vegetation. During periods of railfall, pollution indicators that might be associated with vegetation enter the surface waters by way of storm water drainage.

Wilson et al.(*99*) found the coliform content of grass, hay, and straw to be relatively small except in samples subjected to soil contamination. Thomas and McQuillin(*100*) reported these microorganisms abundant in grass from both ungrazed herbage and intensively grazed pasture. Coliform bacteria were reported by Thomas and Hobson(*101*) as normal inhabitants on ears and panicles of cereal crops. They also noted 2.7 percent of the 148 strains were capable of gas production at 44°C. Fraser, Reid and Malcolm(*102*) reported that coliform bacteria were seldom found on foliage of a wide variety of garden plants, trees and shrubs, and field plants. These authors stated that exceptions were the result of possible contamination by insects, animals, or dust. Many of the earlier investigations related to occurrence of coliform bacteria were based on temperatures other than 35° and 45°C and on media or methods lacking selectivity and sensitivity.

Sherman(*103*) reported that *Streptococcus fecalis* and *S. liquefaciens* types were rather common on plants, but gave no further information. Mundt, Johnson, and Khatchikian(*104*) examined leaves, flowers, and shoots of plants grown in cultivated and uninhabited areas for enterococci and coliform bacteria. They reported "the enterococci group" were isolated from 58.5 percent of 106 samples that included 63 plant species, and coliform bacteria were isolated from 67.0 percent of these samples. In another study, Mundt(*105*) found enterococci on 27 percent of the flowers sampled and 6.8 percent of the buds from the same plants. Totals of 34, 32.2, and 10.4 percent, respectively, of the flowers from nonagricultural, agricultural, and grass plants contained enterococci. Data reported on enterococci per gram of corn flowers indicated corn tassel samples contained Most Probable Number (MPN) densities to a maximum of 300,000 per gram. Recently, Mundt(*106*) described the occurrence of enterococci on plants from the Great Smoky Mountains; enterococci were obtained from 14.2 percent of 2,169 flowers and 3.4 percent of 440 samples of leaves, buds, shoots, fruits, and seeds, but he found no evidence of plant-specific species or variants of the enterococci.

These pollution indicators on vegetation may, in part, be derived from insect contact. Steinhaus(*107*) isolated 11 strains of coliform bacteria from the alimentary tract of two Orthoptera, one Hemiptera, one Coleoptera, and two Lepidoptera. *S. fecalis* was isolated in five insect species (one Orthoptera, one Hemiptera, one Homoptera, and two Lepidoptera). Fecal streptococci were reported by West(*108*) to be present in the housefly (*Musca domestica*). Eaves and Mundt(*109*) recovered fecal streptococci from 20 of 26 species of insects.

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* This material has been published previously in *Applied Microbiology* (Reference 66).

The insufficient data available on the quantitative recovery of coliforms, fecal coliforms, and fecal streptococci from vegetation and insects indicated the need to apply such procedures as described in the investigations of Clark et al.(*63*) and Geldreich et al.(*26, 27, 70*). Any review of the possible distribution of these indicator microorganisms should include a study of the bacteria on vegetation and insects and a determination of the percentages of each type that are fecal coliforms or fecal streptococci. This determination would aid in the further evaluation of the occurrence of these bacterial groups as indicators of fecal pollution.

MATERIALS AND METHODS

A total of 152 species of plants was examined. These were grouped in the following categories: flowering heads, buds, and blossoms from garden ornamental plants, field crops, and wild flowers; foliage from garden vegetables, and field crops, ornamentals including trees, shrubs and various grasses plus wild flowers; sphagnum-type peat moss; and indoor-cultivated plants from greenhouses and homes. These specimens were collected from various ecological environments, including fields, woods, gardens, lawns, cultivated farmlands, railroad cuts, roadways, riverbanks, and marshlands within 30 miles of Cincinnati.

The 40 insect samples were collected from areas where the plants had grown, but not necessarily concurrently. The insect samples included 16 Coleoptera, 11 Orthoptera, 7 Hymenoptera, 3 Lepidoptera, 2 Diptera, and 1 Homoptera.

Samples of plant material in minimal amounts of 20 grams were collected in disposable cups. Weighed portions of this material were added to 9 volumes of buffered sterile water and mixed in a blender at high speed for 30 seconds. Insect specimens were collected in 1-gram minimal amounts, diluted with 99 volumes of buffered sterile water, and then mixed in a blender. In both cases, the suspension was permitted to settle for about 15 minutes and then given a final mixing at high speed for 15 seconds.

The suspensions were examined for coliform bacteria by the confirmed and completed tests with the multiple-tube procedures as described in *Standard Methods*(*19*). The fecal coliform test consisted of confirming all positive presumptive tubes in EC broth at 44.5°C (± 0.5). MPN enumerations of the fecal streptococcus group were performed by the tentative test(*19*) with azide dextrose (AD) presumptive broth followed by confirmation of all positive tubes in ethyl violet azide (EVA) medium. All confirmed-positive EVA tubes were verified by a Gram stain preparation. Pour plates with KF streptococcus agar(*91*) were made by isolating colonies to be studied in the subsequent species or group classification for types of streptococci present. All quantitative data were reported as number of organisms per gram of sample.

Possible inhibition of a fecal coliform by the suspensions of vegetation and insect samples was determined by placing paper discs saturated with blender samples on pour plates containing a heavy concentration of an *E. coli* strain. Each plate also contained several paper discs saturated with suitable antibiotics for positive controls. The pour plates were incubated 24 hours at 35°C and inspected for zones of growth inhibition.

A preliminary isolation of the coliform bacteria from the vegetation and insect samples was obtained by the membrane filter procedure. A minimum of 20 to 50 isolated colonies with a typical sheen were transferred to phenol red lactose broth for fermentation tests and verified in brilliant green lactose broth. Streaking on eosin methylene blue (EMB) agar insured cultural purity. All purified strains were then inoculated into EC broth at 44.5°C and into the media necessary for identification and classification by IMViC types (*19*).

RESULTS AND DISCUSSION

The results of the five-tube confirmed and completed tests and the fecal coliform and streptococcal group tests for each plant and insect group are summarized in Table 22. No coliform or fecal coliform bacteria were detected from either indoor-cultivated foliage or sphagnum moss, and only 2.2 coliforms per gram were detected from wild flower plants at the 75-percentile level by the completed test. The upper-quartile values in the completed test for flower heads, foliage from garden vegetables, and ornamentals were, however, 177, 330, and 130 coliforms per gram, respectively. The fecal coliform test values at the 75-percentile level for these three groups were 0.5, 0.2, and 7.9 per gram, respectively. A total of 790 streptococci per gram of flowering heads was detected at the 75-percentile level. Results from the foliage of garden vegetables, ornamentals, and wild flowers were 490, 2,210, and 109 fecal streptococci per gram, respectively, at the upper-quartile level. At the same percentile level, only 0.8 streptococci per gram was found from indoor-cultivated foliage, and less than 10 per gram were detected from sphagnum moss.

In the completed test, data at the 75-percentile level for various insect groups varied from 9.4 million to 4,900 million coliforms per gram. Fecal coliforms at this level ranged from < 20 to 79,000 per gram, and fecal streptococcal results varied from 0.24 million to 4,900 million per gram.

Table 23 shows the results of the biochemical reactions for 1,203 coliform strains. Twenty to 50 strains from each of 16 flower species and 14 foliage specimens are represented. No IMViC type appears to predominate in either the flower or foliage samples. Fecal coliforms made up 10.9 and 18.5 percent of the flower and foliage coliform strains, respectively. Both vegetation sources had 13 different IMViC

TABLE 22. PERCENTILE DISTRIBUTION BY QUARTILE OF MPN VALUES PER GRAM FOR 152 VEGETATION AND 40 INSECT SAMPLES

Sample classification	No. of Samples	Confirmed test			Completed test			Fecal coliform			Fecal streptococci		
		25%	50%	75%	25%	50%	75%	25%	50%	75%	25%	50%	75%
Vegetation													
Flowering heads, buds, blossoms	81	0.2	109	27,800	<2.0	4	177	<0.2	<2.0	0.5	0.5	27	790
Foliage of:													
Garden vegetables	27	18	1,090	5,420	0.9	43	330	<2.0	<2.0	0.2	2.0	35	490
Ornamentals	15	0.7	130	490	0.4	7.0	130	<0.2	<2.0	7.9	<2.0	49	2,210
Wild flowers	11	<0.2	<2.0	490	<2.0	<2.0	2.2	<0.2	<0.2	<0.2	<0.2	33	109
Sphagnum-type peat moss	8	<4	<4	<10	<4	<4	<10	<4	<4	<10	<4	<4	<10
Indoor-cultivated foliage	10	<0.2	0.5	2.3	<0.2	<0.2	<2	<0.2	<0.2	<0.2	<0.2	0.2	0.8
Insects													
Coleoptera (beetles)	16	8,800	1,084,000,000	2,210,000,000	8,800	94,000,000	490,000,000	<36	<20	79,000	920,000	1,024,000,000	4,900,000,000
Orthoptera (grass-hoppers, etc.)	11	172,000	330,000	34,000,000	1,200	33,000	9,400,000	<20	<20	230	70,000	790,000	3,300,000
Hymenoptera (bees, wasps, and ants)	7	120	1,090,000	27,800,000	80	42,600	27,800,000	<20	<20	140	<20	49,000	240,000,000
Diptera-Homoptera (flies and leafhoppers)	3	1,300	3,200,000	15,800,000	1,300	852,000	15,800,000	<20	<40	<40	700	344,000	3,200,000
Lepidoptera (moths and butterflies)	3	130	490	54,000,000,000	<20	<20	4,900,000,000	<20	<20	<20	20	1,720	240,000

TABLE 23. OCCURRENCE OF COLIFORM TYPES ON VEGETATION

Coliform type	Flowers		Foliage		Summary	
	No. of strains examined	Percent occurrence	No. of strains examined	Percent occurrence	No. of strains examined	Percent occurrence
+ + − −	59	8.4	69	13.7	128	10.6
− − + +	88	12.6	149	29.6	237	19.7
− + − −	0	—	23	4.6	23	1.9
+ + + −	1	0.1	1	0.2	2	0.2
− + − +	101	14.5	67	13.3	168	14.0
+ + − +	96	13.7	20	4.0	116	9.6
− + + +	20	2.9	12	2.4	32	2.7
+ + + +	168	24.0	123	24.4	291	24.2
+ − + +	81	11.6	7	1.4	88	7.3
− − − +	66	9.4	21	4.2	87	7.2
− + + −	1	0.1	4	0.8	5	0.4
− − + −	12	1.7	7	1.4	19	1.6
+ − + −	2	0.3	0	—	2	0.2
+ − − +	4	0.6	1	0.2	5	0.4
+ − − −	0	—	0	—	0	—
Total	699	—	504	—	1,203	—
EC positive	76	10.9	93	18.5	169	14.1

strains, of which 12 were common to both. A total of 76.4 percent of all coliforms found on flowers was represented by five types. Four types comprised 81 percent of all coliform strains examined from foliage specimens. The remaining coliform types for flowers and foliage occurred in varying percentages of less than 10 percent.

A compilation by IMViC types and fecal coliform composition for plants and insects is given in Table 24. Data on vegetation are a consolidation of the foliage and flower results of Table 23, since there was little difference in coliform type composition between the two groups. Note that in both classes of samples there is little contribution from those organisms, i.e., fecal coliforms derived from warm-blooded animals. Specifically, the percentage of fecal coliform strains detected on vegetation and insects was 14.1 and 14.9, respectively.

No coliform type predominated in either the vegetation or insect classes. Five IMViC types in the vegetation class made up 78.1 per-

TABLE 24 COLIFORM DISTRIBUTION BY IMVIC TYPES AND ELEVATED TEMPERATURE (FECAL) TEST

IMViC type	Vegetation coliform types				Insect coliform types			
	No. of strains examined	Percentage isolated	Positive EC tests		No. of strains examined	Percentage isolated	Positive EC tests	
			No. of strains	Percentage positive			No. of strains	Percentage positive
+ + — —	128	10.6	120	93.8	134	12.4	129	96.3
— — + +	237	19.7	15	6.3	113	10.4	1	0.9
— + — —	23	1.9	11	47.8	0	X[a]	0	0.0
+ + + —	2	0.2	1	X[a]	0	X[a]	0	0.0
— + — +	168	14.0	4	2.4	332	30.6	27	8.1
+ + — +	116	9.6	3	2.6	118	10.9	5	4.2
— + + +	32	2.7	0	0.0	28	2.6	0	0.0
+ + + +	291	24.2	0	0.0	254	23.4	0	0.0
+ — + +	88	7.3	0	0.0	46	4.2	0	0.0
— — — +	87	7.2	15	17.2	42	3.9	0	0.0
— + + —	5	0.4	0	0.0	0	X[a]	0	0.0
— — + —	19	1.6	0	0.0	0	X[a]	0	0.0
+ — + —	2	0.2	0	0.0	0	X[a]	0	0.0
+ — — +	5	0.4	0	0.0	8	0.7	0	0.0
+ — — —	0	X[a]	0	0.0	9	0.8	0	0.0
Total	1,203		169	14.1	1,084		162	14.9

[a] Insufficient number of cultures studied.

cent of the coliform strains, with the other 21.9 percent scattered among nine types. Among the insect coliforms examined, five types composed 87.7 percent of all IMViC strains. Five other strains composed the remaining 12.2 percent.

In this study, only 65 vegetation samples, or 42.8 percent of the 152 specimens examined, and 23 insect samples, or 57.5 percent of the 40 insect collections, contained sufficient streptococci to provide isolates for biochemical study. The results of these biochemical tests for the streptococci found on vegetation and insects are given in Tables 25 and 26, respectively. When streptococci were isolated, the predominant organisms grew at both 45° and 10°C; i.e., 87.8 percent of 646 streptococci from vegetation and 95.1 percent of the 226 strains from insects were in this category. No strains capable of growth at 45° but not at 10°C were found on insects. Among streptococci from vegetation, however, 8.8 percent or 57 strains were capable of growth at 45° but not at 10°C. Streptococci that grew at 10° but not at 45°C composed 3.4 percent of total strains isolated from vegetation and 4.8 percent of those from insects.

Results obtained in this study suggest that typical coliforms of the warm-blooded-animal gut, as measured by the fecal coliform test, contributed a relatively small percentage of the organisms associated with vegetation and insects. Fraser et al.(*102*) suggested that most of the coliforms (which were *Aerobacter cloacae*) found on grains are of insect origin. Eaves and Mundt(*109*) demonstrated that insects could transfer streptococci in the environment to the flowers of various grasses, cereals, and nonagricultural plants. They also stated that pollution from wild animals was a remote possibility because of their very low populations in the cultivated fields used in the experiments. Both groups believed that soil was not a major contributor, since soil samples surrounding the plants did not have the coliform or streptococcal composition found on plants.

Fraser et al.(*102*) observed no definite predominant types on plants or on insects. Our data support this view and are in marked contrast to the results previously obtained for warm-blooded animals and soil. The similarity of IMViC types recovered from vegetation and insects suggests their intimate association. Mundt(*105*) has suggested that the enterococci are temporary residents on plants and are disseminated among plants by several different agents, but principally by insects. Apparently, from strong circumstantial evidence, much the same case can be made for the coliform contribution. The low recoveries of fecal coliforms might imply that vegetation and insects are unsuitable environments. None, however, of the 152 vegetation or 40 insect samples exhibited an inhibitory effect on the fecal coliform strain used in the disc sensitivity test. Thus, the possibility of fecal coliform suppression appears untenable. Certainly the contribution from warm-blooded-animal pollution is a minor factor in both vegetation and the dissemination agent, insects.

Quantitatively, the results in Table 22 indicate that warm-blooded-animal contamination, as measured by the fecal coliform

TABLE 25. BIOCHEMICAL REACTIONS OF 646 STREPTOCOCCI ISOLATED FROM VEGETATION[a]

No. of strains	Growth at		Growth reaction in		Reduction of		Final pH in 1% glucose	Hydrolysis of starch[b]	Peptonization of litmus milk[c]
	10°C	45°C	6.5% NaCl	pH 9.6 broth	Methylene blue (0.1%)	Potassium tellurite (1:2,500)			
219	+	+	+	+	+	+	4.1 — 4.5	0	0
80	+	+	+	+	+	+	4.1 — 4.5	0	+
119	+	+	+	+	+	+	4.0 — 4.7	+	0
71	+	+	+	+	+	+	4.0 — 4.7	+	+
5	+	+	+	+	+	+	5.0 — 5.1	0	0
4	+	+	+	+	0	+	4.2 — 4.3	0	0
1	+	+	+	+	0	+	4.3	0	+
1	+	+	+	+	0	0	4.4	0	0
1	+	+	+	+	+	0	4.3	0	0
1	+	+	+	+	0	+	4.5	+	0
1	+	+	+	0	+	+	4.8	+	0
11	+	+	0	+	+	0	4.2 — 4.5	0	0
8	+	+	0	+	+	+	4.3 — 4.4	0	0
22	+	+	0	+	+	+	4.5 — 5.3	+	0
1	+	+	0	+	+	+	4.3	+	+
14	+	+	0	0	+	+	4.3 — 4.4	0	0
4	+	+	0	0	+	0	4.9 — 5.0	0	0

2	+	+	0	0	0	0	4.9	0	0
2	+	+	0	+	0	0	4.9 — 5.0	+	0
7	0	+	+	+	+	+	4.7 — 5.1	0	0
1	0	+	+	+	+	+	4.5	+	0
6	0	+	0	+	+	+	4.6 — 4.9	0	0
18	0	+	0	+	+	+	4.4 — 5.3	+	0
2	0	+	0	+	+	+	4.5	+	+
2	0	+	0	0	+	+	4.4 — 4.5	+	0
1	0	+	0	0	+	0	4.5 — 5.2	+	0
16	0	+	0	0	+	0	4.6 — 5.3	0	0
4	0	+	0	0	0	0	4.6 — 5.1	0	0
10	+	0	+	+	+	+	4.1 — 4.5	0	0
4	+	0	+	+	0	+	4.1	0	0
2	+	0	0	+	+	+	4.3	0	0
6	+	0	0	0	+	+	4.3 — 4.4	0	0

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[a] All strains were catalase-negative; all strains grew in 40% bile broth.

[b] A total of 241 strains hydrolyzed starch (37.7%).

[c] A total of 155 strains peptonized litmus milk (24.0%).

TABLE 26. BIOCHEMICAL REACTIONS OF 226 STREPTOCOCCI ISOLATED FROM INSECTS[a]

No. of strains	Growth at		Growth reaction in		Reduction of		Final pH in 1% glucose	Hydrolysis of starch	Peptonization of litmus milk[b]
	10°C	45°C	6.5% NaCl	pH 9.6 broth	Methylene blue (0.1%)	Potassium tellurite (1:2,500)			
89	+	+	+	+	+	+	4.0 — 4.7	0	0
102	+	+	+	+	+	+	4.0 — 4.5	0	+
13	+	+	+	+	0	+	4.0 — 4.7	0	0
2	+	+	+	+	+	+	4.2	0	0[c]
1	+	+	+	+	0	+	4.8	0	+
1	+	+	+	+	+	0	4.0	0	0
3	+	+	+	0	+	+	4.0 — 4.6	0	0
2	+	+	0	+	0	+	4.3 — 4.7	0	0
2	+	+	0	+	0	0	4.3 — 4.4	0	0
3	+	0	+	+	+	+	4.2 — 4.6	0	0
1	+	0	+	+	+	0	4.1	0	0
1	+	0	0	+	0	0	4.4	0	0
4	+	0	0	+	0	+	4.5 — 4.7	0	0
2	+	0	+	+	0	+	4.6	0	0

[a] All strains were catalase-negative; all grew in 40% bile broth.

[b] Of 226 strains, 103 (45.6%) peptonized milk.

[c] Arginine hydrolysis-negative, all other arginine tests performed positive with these exceptions.

test, is usually not observed. The higher levels of streptococci reflect either their more frequent occurrence on vegetation or the inclusion of streptococcal types not of warm-blooded-animal origin. Mundt et al.(*104*) reported 58.5 percent of 106 plant samples contained enterococci. One possible reason for this divergence of fecal coliform from streptococcal values may be found in an inspection of the MPN values for insects (Table 22). The high densities of streptococci and relatively low fecal coliform levels on insects are repeated in a similar pattern but at lower density levels on vegetation.

These high streptococci levels may possibly result from some proliferation on insects. Mundt, Coggins, and Johnson(*110*) demonstrated that *S. fecalis* var. *liquefaciens* can establish commensal growth on plants of beans, corn, rye, and cabbage. A similar adaptation to the insect environment could result in some multiplication of the streptococci and may account for the extremely high values found on the *Coleoptera* (beetles). Another factor that might have caused such high counts is in the streptococcus detection procedure. AD-EVA streptococcal medium will support the growth of *S. lactis*(*91*). Stark and Sherman(*111*) reported this organism on corn, navy beans, cabbage, wheat, garden peas, and head lettuce. *S. lactis* was also reported by West(*108*) to be associated with the housefly, *Musca domestica*, and by Eaves and Mundt(*109*), with various *Hymenoptera, Coleoptera*, and one species of *Neuroptera*.

Of the 646 strains isolated from vegetation, 241 (37.7%) hydrolyzed starch, even though all other criteria were typical of the enterococcus group as defined by Sherman(*103*). These results concur with those of Langston and Bouma(*112*) who studied microorganisms of grass silage. They found that a majority of the streptococci with *S. fecalis* characteristics also hydrolyzed starch. In our studies of fecal streptococci from water, warm-blooded animals, cold-blooded animals, insects, and soil, we have never found *S. fecalis* strains displaying this characteristic.

Peptonization of milk appears to be a relatively common characteristic of fecal streptococcal strains isolated from insects; 45.6 percent were positive, as opposed to 24.0 percent of strains isolated from vegetation. In our study of 3,158 strains isolated from feces of man and warm-blooded animals, 16.9 percent or 533 fecal streptococcal cultures peptonized milk. Since the percentage of peptonizing strains from insects is nearly three times that of strains from humans and other warm-blooded-animal feces, the peptonizing characteristic may be important in studies of streptococcal strains from insects.

Data presented here indicate that though there were some coliform bacteria on many of the vegetation samples examined, coliforms present were fewer in numbers than those reported in the literature. This discrepancy can be partly explained with a completed coliform test to eliminate the many anaerobic and aerobic sporeforming bacteria, and by geographical differences in the environments of the various studies reported. These sporeforming bacteria can be the cause of many false positive results in the brilliant green lactose broth confirmatory test. Such interferences were also observed in the confirmed coliform test in a study of soil samples from various geographical areas. Use of a Completed MPN Test is essential, therefore, in all studies of coliform populations on vegetation.

These results confirm other reports that the numbers of coliforms, fecal coliforms, and fecal streptococci on plants are very low. They also show that the ratio of fecal coliforms to coliforms is quite small. Apparently, significant numbers of these indicator organisms are not removed from vegetation by rainfall and carried away in the runoff. Our findings support the current interpretation that fecal coliforms in surface waters are largely, if not completely, derived from fecal pollution of animal origin.

CHAPTER VI. SOILS*

Soil may contribute large numbers of bacteria to drainage water. The number and species present depend on such factors as soil type, erosion, land use, and amount of rainfall. The possibility that soil may add to the coli-aerogenes content of water has been of considerable interest and speculation in sanitary bacteriology. Much controversy still exists concerning the distribution, origin, and sanitary significance of these bacteria.

Johnson and Levine(*113*), Chen and Rettger(*114*), Koser(*115*), Raghavachari(*116*), and Frank and Skinner(*117*) have reported *Aerobacter aerogenes* to be the predominant coli-aerogenes type in soils, with percentages varying from 45.0 to 93.5. The intermediate type giving IMViC reactions of $^{-+-+}$ was found by Bardsley(*118*) to be dominant in soil. Taylor(*119*) considered *Bacterium coli* type I the most prevalent type regardless of location or soil type. Randall(*120*) stated that the proportion of *B. coli* type I was directly related to the degree of probable pollution of the soil, and that the proportion of intermediates increased with remoteness of the soil from potential polluting agents.

All coli-aerogenes bacteria found in soil were thought by Parr (*121*) to be of fecal origin. Griffin and Stuart(*52*) suggested that only *Escherichia coli* was derived from feces. Taylor(*119*) found insufficient evidence to conclude that any of the coli-aerogenes types studied by him were normal soil inhabitants. *Standard Methods*(*19*) suggest that coli-aerogenes bacteria (designated as the "coliform group" in the United States) are probably not normal inhabitants of soil. It is assumed that these authors are considering only the coli-aerogenes group as defined in the practice of sanitary bacteriology and not the many bacteria that resemble these organisms, but which are unable to grow at 35°C.

These conflicting observations concerning the distribution and origin of coli-aerogenes types may in part be the result of variability of soil types and their associated environments. In addition, technical laboratory procedures have varied from enrichment in broth to direct streaks on selective agar media, from glucose to lactose fermentation, and from incubation at 30° to 44°C. As a consequence, the resulting type distribution pattern would be influenced by whatever segment of the coli-aerogenes population might be favored by a particular growth and identification procedure.

Moreover, practically all the investigations have depended upon the primary isolation of coli-aerogenes bacteria in liquid media; such a procedure is not applicable to the quantitative estimation of various types of these organisms, however, as overgrowth by one or more types results in the suppression of minority organisms.

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* This material has been published previously in *Journal Applied Bacteriology*, Reading, England (Reference 27).

Recently, Clark et al.*(63)* and Geldreich et al.*(26, 70)* used the membrane filter technique for the study of the distribution patterns of coli-aerogenes organisms, and the elevated temperature test for the detection of the fecal types. These studies of the distribution of coli-aerogenes bacteria in water and fecal samples suggested that the two procedures may offer an improved method for soil investigation and avoid past deficiencies in procedure.

MATERIALS AND METHODS

Sources and Types of Samples

In this investigation, 251 soils from various geographical areas in 26 States and three foreign countries were examined; these were grouped in the following classes: arid soils, which included samples from sand dunes, an alpine area, and a salt flat; subterranean soils, consisting of samples from caves and a coal mine; submerged soils, collected from creek, river and lake bottoms and sludge deposits, and a submerged salt-water beach soil; pasture land samples, from mountain pastures, sagebrush and mesquite open ranges, mesas, prairie land, blue-grass field and farm-livestock pastures; shore-line samples, collected from soils adjacent to various creeks, rivers reservoirs, lakes, and oceans and including also samples from swamps, bogs, and marshlands; woodland samples, taken from national forests, state parks, and farm woods; inhabited soils, collected from residential lawns, city parks, lawns adjacent to public buildings, institutions and industrial plants, excavated soil, bird-nesting areas, an animal burrow, and a rural camping location; cultivated soils, which included wheat, oats, maize, and beet fields, tomato, strawberry and melon patches, greenhouse soils, an apple orchard, and a compost heap; fallow fields, plowed fields, and irrigated farm soil; and polluted soils, sampled from various soil classes known to be in contact with recent contamination.

Sampling Procedure and Examination of Samples

The samples were collected by scraping away approximately 1 inch of surface soil and filling a sterile 1-ounce ointment tin with the soil under the denuded area. Samples of 10 grams of each soil suspended in 90 milliliters of buffered sterile water were examined by the confirmed multiple tube procedure for the coli-aerogenes group, the completed test as described in *Standard Methods(19)*, and the fecal coli-aerogenes tests, all of which were based on five tubes at each dilution and gave results as most probable numbers (MPN). The fecal test was accomplished by confirming presumptive positive tubes in EC broth at 44.5°C (± 0.5). This elevated temperature test has been shown by Geldreich et al.*(26)* to have a 96.3 percent positive correlation with coli-aerogenes strains found in warm-blooded animals. The membrane filter technique was used to effect a preliminary isolation of the organisms. A minimum of 25 to 50 isolated colonies with a typical sheen were transferred to phenol red lactose broth for fermentation tests, verified in brilliant green lactose bile broth, and

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then streaked on eosin methylene blue agar (EMB) to ensure cultural purity. The strains were then ready for simultaneous inoculations into boric acid lactose broth (BALB) and EC broth to test for fermentation at 43° and 45°C, respectively, and also into the necessary media for identification and classification by the IMViC tests. The elevated temperature procedures were those described by Vaughn et al.(*50*) for BALB and a modification of the Hajna and Perry(*62*) EC test. The IMViC procedures used were those described in *Standard Methods*(*19*).

RESULTS

The results of the five-tube confirmed, completed, and elevated temperature (fecal coli-aerogenes bacteria) tests for each of the soil classes are summarized in Table 27. In the completed test, many samples from the arid, subterranean, submerged, pasture, and shore-line soil classes were found to contain < 1.8 coli-aerogenes bacteria per gram at the 25-percentile point. These organisms were not detected in any of the seven arid samples. The upper-quartile values, respectively, for the completed test were 12 and 13 coli-aerogenes bacteria per gram for subterranean and submerged samples, and 170 and 430 for pasture and shore-line soils. The 25- to 75-quartile ranges for woodland, inhabited, and cultivated soils were 1.8 to 2,900; 8.3 to 2,200; and 8.1 to 1,800 per gram respectively. The density for polluted samples ranged from 9,500 to 220,000 per gram. In the fecal test, the fecal coli-aerogenes content of all eight unpolluted soil classes was very low, with the majority of samples indeterminate (i.e. < 1.8 per gram). The number of fecal coli-aerogenes organisms for polluted samples ranged from 3,300 to 49,000 per gram for the 25- to 75-percentile points.

The results of repeated sampling from four soils during irregular intervals over an 8- to 10-month period are reported in Table 28. The results of the completed tests for samples from the oat field varied from 14 to 4,600 per gram and those for the mountain meadow from 6.1 to 17,000 per gram. A variation of < 1.8 to 250 per gram occurred in the counts of samples from Flynn Creek timberland. The results for Needle Creek timberland were in the range of < 1.8 to 26 per gram. The fecal coli-aerogenes results were consistently low, and 15 of the 26 MPN values were < 1.8. The highest fecal coli-aerogenes density of 110 was found in one oat field sample; the next two highest were 45 and 33.

During this investigation 3,013 strains of coli-aerogenes organisms were examined. This represented 20 to 100 strains from each of 54 undisturbed soils and 13 polluted soils. The predominant IMViC types in the undisturbed soils were − + − + (48.1 percent) and − − + + (18.8 percent). Seven other types occurred with a frequency greater than 2 percent. With reference to the polluted soils, 98.9 percent of the total coli-aerogenes organisms were represented by four types (+ + − −, − + − +, + + − + and − − + +). No single type in the occasionally occurring groups from either undisturbed or polluted soils constituted more than 1 percent of the coli-aerogenes content.

TABLE 27. THE PERCENTILE DISTRIBUTION OF MPN VALUES PER GRAM OF SAMPLE FOR 251 SOILS

Soil class	No. of samples	Counts (MPN/g) in quartile grades according to								
		Confirmed test			Completed test			Fecal test		
		25%	50%	75%	25%	50%	75%	25%	50%	75%
Arid	7	< 1.8	< 1.8	< 1.8	< 1.8	< 1.8	< 1.8	< 1.8	< 1.8	< 1.8
Subterranean	9	< 1.8	2.0	230	< 1.8	< 1.8	12	< 1.8	< 1.8	< 1.8
Submerged	14	< 1.8	4.5	13	< 1.8	2.0	13	< 1.8	< 1.8	13
Pasture	23	< 1.8	150	2,500	< 1.8	20	170	< 1.8	3.0	17
Shore line	58	< 1.8	200	3,300	< 1.8	75	430	< 1.8	< 1.8	49
Woodland	56	6.8	200	14,000	1.8	17	2,900	< 1.8	< 1.8	11
Inhabited	21	33	2,600	33,000	8.3	84	2,200	< 1.8	< 1.8	18
Cultivated	35	170	3,000	13,000	8.1	150	1,800	< 1.8	< 1.8	11
Polluted	28	23,000	64,000	330,000	9,500	48,000	220,000	3,300	11,000	49,000

TABLE 28. THE VARIATION IN COUNTS OF COLI-AEROGENES BACTERIA OBTAINED ON REPEAT SAMPLES OF SOILS FROM FOUR SOURCES

Source of sample	Interval between samples, days	Counts (MPN/g) obtained in Confirmed test	Counts (MPN/g) obtained in Completed test	Counts (MPN/g) obtained in Fecal test
Oat field	0	4,600	4,600	110
	16	700	220	11
	26	170,000	170	45
	17	2,200	17	< 1.8
	144	7,900	14	< 1.8
Mountain meadow	0	79	13	7.8
	15	700	64	1.8
	51	15,000	6.1	< 1.8
	61	7,900	1,400	11
	144	17,000	17,000	< 1.8
Timberland (Flynn Creek)	0	11	11	11
	15	1.8	< 1.8	< 1.8
	14	49	49	< 1.8
	37	220	13	< 1.8
	21	3,500	31	< 1.8
	33	84	46	33
	17	13,000	250	4.5
	144	1.8	< 1.8	< 1.8
Timberland (Needle Branch)	0	7.8	7.8	7.8
	15	< 1.8	< 1.8	< 1.8
	14	< 1.8	< 1.8	< 1.8
	37	7.8	7.8	< 1.8
	21	< 1.8	< 1.8	< 1.8
	33	70	26	< 1.8
	17	11,000	7.3	< 1.8
	144	6.8	4.5	2.0

DISCUSSION

The results of the confirmed EC elevated temperature procedure indicate that fecal coli-aerogenes bacteria are usually absent in undisturbed soils, or are present only in comparatively small numbers. There was, however, a sharp increase in the numbers of these types from soils known to be polluted. This is in agreement with a study by Allen, Grindley, and Brooks(*122*) of bottom deposits from lakes and estuaries. Their elevated temperature test confirmed all positive MacConkey's broth presumptive tubes (37°C) in the same medium at 44°C. Randall(*120*) modified this technique by confirming in MacConkey's agar roll tubes at 44°C. He concluded that the density of *E. coli* in soil was directly proportional to the degree of probable pollution. Thus, three different elevated temperature procedures have supported the same conclusion about the occurrence of fecal coli-aerogenes bacteria in soil; i.e., the more remote an area from human and animal pollution, the less likely fecal types will be found.

Intermediates have been reported frequently to constitute a considerable proportion of the coli-aerogenes bacteria in soils. In this study, 48.1 percent of 2,348 strains from 54 undisturbed soils were of

TABLE 29. THE OCCURRENCE OF DIFFERENT IMViC TYPES OF COLI-AEROGENES BACTERIA IN 67 SAMPLES OF SOIL

IMViC type	Strains isolated from			
	Undisturbed soils[a]		Polluted soils[b]	
	No.	%	No.	%
+ + − −	131	5.6	536	80.6
− − + +	443	18.8	13	2.0
− + − −	78	3.3	1	0.2
+ + + −	7	0.3	0	0
− + − +	1,131	48.1	87	13.0
+ + − +	87	3.7	22	3.3
− + + +	181	7.7	5	0.7
+ + + +	159	6.8	0	0
+ − + +	67	2.9	0	0
− − − +	4	0.2	1	0.2
− + + −	1	[c]	0	0
− − + −	53	2.3	0	0
+ − + −	6	0.3	0	0

[a] 54 soils were examined and **2,348** cultures isolated and studied; 216 (9.2%) and 200 (8.5%) of the cultures were positive in EC and BALB media, respectively.

[b] 13 soils were examined and 665 cultures isolated and studied; 551 (82.9%) and 545 (82.0%) of the cultures were positive in EC and BALB media, respectively.

[c] Insufficient cultures studied.

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an intermediate type giving the IMViC reaction −+−+ (Table 29). In a study of a protected raw water supply for a large western city, also, this type comprised 51.6 percent of 289 strains recovered. Since the entire area was enclosed by a fence, birds or small rodents were probably the sources of the 1.04 percent of fecal coli-aerogenes bacteria found. Thus, the origin of the high percentage of IMViC type −+−+ in this raw water supply was probably the soil along the watershed. In our study, intermediate strains occurred in 75.6 percent of the 2,348 cultures isolated from undisturbed soils, whereas intermediate types comprised only 17.4 percent of the 665 strains from polluted soils. In the protected raw water supply of the western city cited, 93.1 percent of the 289 strains were intermediate in type, giving IMViC results between ++−− and −−++.

These data provide strong circumstantial evidence that the IMViC −+−+ type of coli-aerogenes organism is predominantly a soil type. This conclusion is based not only upon the results of the present study, in which 48.1 percent of the strains isolated was such a type, but also on the very low occurrence of IMViC type −+−+ (0.6 percent) in fecal samples described by Geldreich et al. (*26*), and the occurrence of this type in certain mountain streams studied at various times in the past.

Since soil abounds with many anaerobic and aerobic sporeforming bacteria, and symbiosis is common, to observe large discrepancies in confirmed and completed tests is not unusual. The largest deviation occurred in a soil collected from a creek bank, for which the confirmed MPN value was 1.3 x 10^6 per gram, whereas the completed MPN result was only 270 per gram. It thus appears that the combination of the chemical agents, lauryl sulphate in the presumptive broth and brilliant green plus bile salts in the confirmatory medium, was ineffective in suppressing the growth of some bacteria that were not coli-aerogenes organisms. Because of this frequency of false positive reactions, the completed test must be used in preference to the confirmed procedure in the examination of soils.

CHAPTER VII. WATER

The data presented thus far have demonstrated the elevated temperature test to be a highly specific measurement of the coliform organisms derived from the gut of warm-blooded animals. With the excrement, waste effluents bring to the stream fecal coliform bacteria that can readily be detected quantitatively. The presence of these bacteria indicates that a potential health hazard exists for various multiple uses of such water resources.

In this chapter, the significance of fecal coliforms in various surface waters subject to pollution is discussed. The occurrence of any coliform bacteria, fecal or non-fecal, in treated water should not be tolerated. Thus, the value of the fecal coliform test lies in its application to investigations of stream pollution, sewage treatment systems, and bathing water quality.

DRINKING WATER*

The coliform group, as specified in USPHS Drinking Water Standards(*123*), is defined in *Standard Methods*(*19*): "The coliform group includes all of the aerobic and facultative anaerobic, gram-negative, nonsporeforming, rod-shaped bacilli which ferment lactose with gas formation within 48 hours at 35°C."

Organisms in the coliform group differ in biochemical and serologic characteristics and in their natural sources and habitats. *Escherichia coli* characteristically inhabits human and animal intestines (*1, 15, 81, 124*). *Aerobacter aerogenes* and *Aerobacter cloacae* are frequently found on various types of vegetation(*16, 100, 101*) and in materials used in joints and valves of pumps and in pipelines(*125, 126*). The intermediate-aerogenes-cloacae (IAC) subgroups may be found in fecal discharges, but usually in smaller numbers than *E. coli*. *A. aerogenes* and intermediate types of organisms are commonly present in soil(*114, 117, 118*) and in waters polluted sometime in the past. Another subgroup comprises plant pathogens(*127*) and other organisms of indefinite taxonomy about whose habitat information is limited. All the subgroups may be found in sewage and in polluted waters. *E. coli* is therefore frequently referred to as fecal coli; the IAC group as non-fecal. These terms, however, are only relative.

Available information indicates that organisms of the IAC group tend to survive longer in water than do fecal coliform organisms (*128-130*). The IAC group also tends to be somewhat more resistant to chlorination than *E. coli* or the commonly occurring bacterial intestinal pathogens(*131-134*). Because of these and other reasons, the relative survival times of the coliform subgroups may be useful in distinguishing recent from less recent pollution. In waters recently

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* This material was published previously in *Journal American Water Works Association* (Reference 69).

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contaminated with sewage, fecal coliform organisms are expected to be present in greater numbers than those of the IAC subgroup; but in waters that have been contaminated for a considerable time or have been insufficiently chlorinated, organisms of the IAC subgroup may be more numerous than fecal coliform organisms.

Because various members of the coliform group normally grow in diverse natural habitats, attempts have been made to differentiate the population in polluted waters according to their original sources (*135, 136, 137*).

Sanitary Significance

Information on the sanitary significance of the various types of coliform organisms is incomplete. In relation to untreated waters, however, the present position may be stated as follows:

Fecal coliform organisms may be considered indicators of recent fecal pollution. No satisfactory method is currently available for differentiating fecal coliform organisms of human and animal origin. Therefore, it is necessary to consider all fecal coliform organisms as indicative of dangerous contamination.

In the absence of fecal coliform organisms, the presence of IAC group organisms in untreated waters may be the result of relatively less recent fecal pollution, soil runoff water, or, infrequently, fecal pollution containing only the IAC group.

In general terms, the presence of fecal coliform organisms indicates recent and possibly dangerous pollution. The presence of IAC organisms suggests less recent pollution or reveals the existence of defects in water treatment or distribution.

Summation

The presence of any type of coliform organism in treated drinking water suggests either inadequate treatment or access of undesirable materials to the water after treatment. Although some differences exist between strain and subgroup organisms with regard to survival under natural conditions and resistance to chlorination, in general all the coliform organisms exhibit survival and resistance patterns in the same order of magnitude. The presence of coliform organisms (as defined earlier) in treated water calls for definitive action for their elimination.

Insofar as bacterial pathogens are concerned, the coliform group is considered a reliable indicator of the adequacy of treatment. As an indicator of pollution in drinking water supply systems, and indirectly as an indication of protection provided, the coliform group is preferred to fecal coliform organisms. Whether these considerations can be extended to include rickettsial and viral organisms has not been definitely determined.

SEWAGE AND STABILIZATION PONDS*

The increasing interest in waste stabilization ponds as an economical method of waste disposal is related, in part, to reportedly high coliform removals. Various publications in recent years have reported coliform removals of 90 percent or more through waste-stabilization-pond treatment in different areas of the country. In a study of 99 communities in the northern plains states, Towne, Bartsch, and Davis(*138*) reported coliform bacterial reductions greater than 95 percent during the four seasons of 1 year. Cooley and Jennings (*139*) stated that between 90- and 95-percent coliform reduction occurred in a sewage stabilization pond at Farmville, Virginia. Even though percent removals were high, 1 to 10 percent of the raw sewage organisms still were being discharged into the receiving stream, representing numerical counts of 4 to 10 million coliforms per 100 milliliters. In a study of a similar system at Fayette, Missouri, Neel, McDermott and Monday(*140*) found reductions were 99 percent during a 10-month period. Sidio, Hartman, and Fugazzotto(*141*) also observed 99-percent reduction in a stabilization pond in Allegheny County, Pennsylvania.

All of these investigations used the confirmed test for coliform organisms as the only bacterial indicator of pollution removal. One of the characteristics of an ideal bacterial indicator is its constant occurrence in human feces and consistent presence in sewage and polluted waters. This indicator must be easily and accurately enumerated and must yield results with minimum incubation. Many bacterial species and groups have been proposed as pollution indicators, but relatively few are in common usage. A large segment of the bacteria present in sewage are without sanitary significance because they are widely distributed in air or soil, come from unknown sources, or have no known or suspected association with human or animal pollution.

Recent interest in the use of the fecal coliform and streptococcus groups as more accurate measurements of warm-blooded-animal pollution has prompted studies of the use of these bacterial systems as indicators of waste-stabilization-pond-treatment efficiency. These indicators are defined as follows: Coliform bacteria include all aerobic and facultative anaerobic gram-negative nonsporeforming rods that ferment lactose with gas production within 48 ± 3 hours at 35°C(*19*). Fecal coliforms are those organisms that ferment lactose with gas production within 24 hours at 44.5°C (± 0.5) and are thus somewhat more inclusive than $^{++--}$ IMViC strain *Escherichia coli*, type 1(*26*). Fecal streptococci include the enterococcus group, *Streptococcus mitis-salivarius* group, *S. bovis* group, *S. equinus* group, and an atypical group that closely resembles the enterococcus group, but with one or more differences in critical biochemical tests(*91*). Included are all streptococcal species that grow in brain-heart infusion broth at 45° and 10°C or at 45°C only, and excluded are those streptococci that grow at 10°C but fail to grow at 45°C. In the application of these definitions, the enterococcus group includes only four species: *S. fecalis*, *S. fecalis* var. *zymogens*, *S. fecalis* var. *liquefaciens*, and *S. durans*. The enterococcus group, thus, probably includes most of the streptococci found in human feces and a portion of those present in the feces of other domestic warm-blooded animals that have been investigated. The fecal streptococcal group, therefore, cannot be considered synonymous with the enterococcal group.

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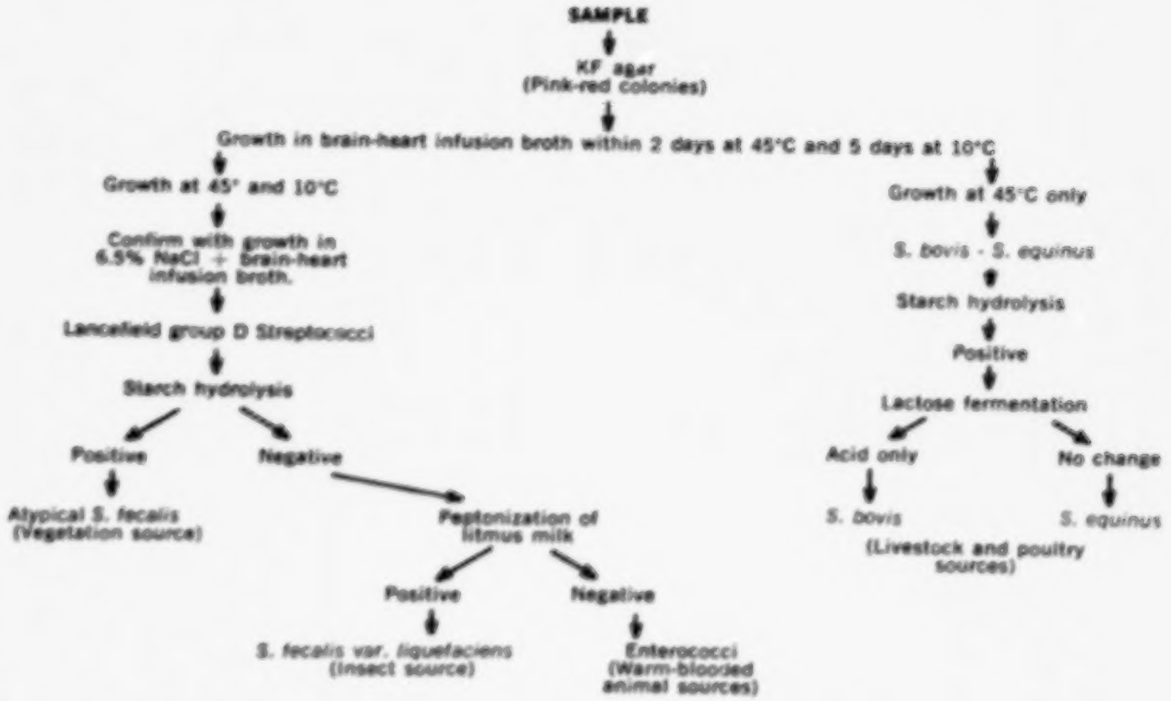
* This material was published previously in *Journal Water Pollution Control Federation* (Reference 152).

Finally, *S. bovis* was used in a study of a waste stabilization pond as a specific indicator of domestic animal pollution. This organism could be separated from the combined fecal streptococci by its abilities to hydrolyze starch and to grow in brain-heart infusion broth at 45°C, but not at 10°C.

Methods

These bacterial pollution indicators were enumerated in our study of raw sewage and effluent from a waste stabilization pond at a state-prison dairy farm near Lebanon, Ohio(*142*), operated by the Ohio Department of Correction and Welfare. Some 200 men work on the farm and live in a dormitory. Sewage from the dormitory plus that of the laundry was diverted into four ¼-acre (0.1-hectare) and one 2-acre (0.8-hectare) waste stabilization ponds. Wastes from the kitchen were diverted to a creek and were not a part of the sewage entering the ponds. This report concerns a study of the raw sewage and effluent from pond 1, one of the ¼-acre ponds. The pond was lined with a thin sheet of plastic to prevent loss of water by seepage through the bottom of the dirt dikes. Pond depth averaged 4 to 5 feet (1.2 to 1.5 m) throughout the year. The pond received a loading from the raw sewage of approximately 50 pounds (23 kg) BOD per day.

An intensive 10-day survey was made during each of the four seasons of the year. Samples were collected every 2 hours and composited every 4 hours into a single bacteriological sample. Each 10 days constituted a single study period with 60 samples of sewage and pond effluent. Such a sampling schedule was found essential because of the fluctuations in densities of indicator microorganisms in both sewage and pond effluent during a 24-hour period. The coliform examination was made by the multiple-tube method with five replicates in three significant decimal dilutions, as described for the Confirmed MPN Procedure in *Standard Methods*(*19*). The fecal coliform MPN consisted of confirming all positive presumptive lactose tubes in EC medium for lactose fermentation at 44.5°C (± 0.5) in 24 hours. The streptococcal direct-count membrane filter method included use of the KF medium described by Kenner, Clark, and Kabler (*143*). Additionally, isolated colonies were picked from low-density KF plates, their cultural purity determined, and further biochemical studies performed on them to identify the fecal streptococci as given in Figure 4.


SAMPLE
KF agar
(Pink-red colonies)
Growth in brain-heart infusion broth within 2 days at 45°C and 5 days at 10°C
Growth at 45° and 10°C
Confirm with growth in 6.5% NaCl + brain-heart infusion broth.
Lancefield group D Streptococci
Starch hydrolysis
Positive
Negative
Atypical S. fecalis
(Vegetation source)
Peptonization of litmus milk
Positive
Negative
S. fecalis var. liquefaciens
(Insect source)
Enterococci
(Warm-blooded animal sources)
Growth at 45°C only
S. bovis - S. equinus
Starch hydrolysis
Positive
Lactose fermentation
Acid only
No change
S. bovis
S. equinus
(Livestock and poultry sources)

Note: A small percentage of unclassified strains (biotypes) will be found since fecal streptococci vary greatly in their biochemical reactions.

Figure 4 — Schematic outline for identification of fecal streptococci.

Results

No important differences in the coliform densities for raw sewage were noted during spring, summer, and autumn (Table 30). A slight reduction in numbers of coliforms occurred during the winter. Fecal coliform densities were between 790,000 and 920,000 per 100 milliliters for three seasons; in autumn, the fecal coliforms increased to 2.65 million per 100 milliliters. Fecal streptococcal densities in the sewage varied from 2.1 to 4.1 million per 100 milliliters.

TABLE 30. BACTERIAL REDUCTION IN POND EFFLUENT[a]

Season and test	Sewage density[a]	Pond effluent	
		Density[a]	Reduction, %
Confirmed coliform test			
Spring	3.20	0.265	91.8
Summer	3.30	0.350	89.4
Autumn	3.30	0.185	94.4
Winter	2.40	0.340	85.9
Fecal coliform test			
Spring	0.92	0.049	94.7
Summer	0.81	0.014	98.3
Autumn	2.65	0.071	97.4
Winter	0.79	0.096	87.9
Fecal streptococci test			
Spring	4.10	0.010	99.8
Summer	2.10	0.017	99.2
Autumn	3.40	0.098	97.2
Winter	2.70	0.082	97.0

[a] Bacteria in million/100 ml calculated as a geometric mean density.

The waste stabilization pond effected a reduction in total coliform population from a minimum of 85.9 percent in winter to a maximum of 94.4 percent in autumn. Fecal coliform reductions were between 87.9 and 98.3 percent. The greatest reduction occurred in fecal streptococci, which were reduced from 97.0 to 99.8 percent during the four seasons.

The potential pollution indicator loadings of this prison sewage and pond effluent were calculated on the basis of the number of bacteria per 24 hours of discharge (Table 31). For the summer period,

TABLE 31. BACTERIAL DISCHARGES IN SEWAGE AND POND EFFLUENT PER 24 HOURS

Season	Bacterial test	Sewage flow, gal[a]	Bacteria discharge, million	Pond effluent, gal[a]	Bacteria discharge, million
Spring		33,000		8,300	
	Total coliform		4,000,000		83,000
	Fecal coliform		1,200,000		15,000
	Fecal streptococci		5,100,000		3,100
Summer		42,000		11,000	
	Total coliform		5,300,000		150,000
	Fecal coliform		1,300,000		6,000
	Fecal streptococci		3,400,000		7,300
Autumn		32,000		8,400	
	Total coliform		4,100,000		59,000
	Fecal coliform		3,300,000		22,000
	Fecal streptococci		4,200,000		31,000
Winter		42,000		15,000	
	Total coliform		3,800,000		190,000
	Fecal coliform		1,300,000		55,000
	Fecal streptococci		4,300,000		47,000

[a] Gal x 0.00379 = cu m.

even with maximum reduction of fecal coliforms in pond effluent of 98.3 percent (Table 30), approximately 6,000 millions were still being discharged into the adjacent creek each 24 hours. This method of pollution calculation is valuable for estimating the quantity of pollution contributed by a given sewage discharge to a receiving stream.

The importance of frequent sampling of sewage during a 24-hour period is shown in Table 32. These data are based on the 10-day geometric mean values for samples collected during the spring study period and represent the relative numbers of bacteria in the raw sewage discharged into the pond, and in the pond effluent. Note that the total coliform densities in the raw sewage reached peaks at 6 AM and noon. Each of these peaks preceded the fecal streptococcal maximum values in the sewage by 2 hours. A considerable fluctuation in the streptococcal densities occurred in untreated sewage, with 8 AM and 2 PM values representing the higher levels. The fecal coliform peaks in the sewage occurred at 8 AM and 6 PM. The 8 AM rise corresponds in time to that for fecal streptococci, but the 6 PM rise is unaccountably 4 hours after the afternoon fecal streptococci peak value. The data for the pond effluent reveal only a slight tendency toward peak bacterial densities during various time periods.

The distribution of fecal streptococci in prison sewage, domestic sewage, and human and cow feces is presented in Table 33. The feces of dairy workers contained only the enterococcus segment, whereas the predominant fecal streptococcus in cows was *S. bovis* (63.0 per-

TABLE 32. POLLUTION INDICATORS[a] PER SAMPLE PERIOD

Sampling time	Total coliforms		Fecal coliforms		Fecal streptococci	
	Raw sewage	Pond effluent	Raw sewage	Pond effluent	Raw sewage	Pond effluent
Midnight + 2 AM + 4 AM	4.60	0.170	1.10	0.033	0.89	0.10
6 AM	5.50	0.200	1.50	0.035	2.40	0.11
8 AM	2.70	0.210	1.60	0.045	14.00	0.14
10 AM + Noon	5.00	0.250	0.78	0.071	3.00	0.16
2 PM	4.00	0.360	0.90	0.059	7.10	0.15
4 PM	2.00	0.130	0.42	0.026	3.50	0.06
6 PM	3.10	0.170	1.30	0.039	3.50	0.06
8 PM + 10 PM	3.80	0.140	1.10	0.052	2.80	0.10

[a] Geometric mean values in million/100 ml.

TABLE 33. DISTRIBUTION OF FECAL STREPTOCOCCI IN SEWAGES AND IN HUMAN AND COW FECES

	Number of cultures	Enterococcus, %	*S. bovis* %	*S. equinus acidominius*, %	*S. mitis salivarius*, %	*S. anginosus*, %
Prison sewage (laundry days)	332	54.0	41.1	4.9	None	None
Prison sewage (non-laundry days)	250	96.0	4.0	None	None	None
Human feces (10 dairy workers)	500	100.0	None	None	None	None
Cow feces (12 samples)	438	28.0	63.0	9.0	None	None
Domestic sewage (from a suburb of Cincinnati, O.)	200	97.5	0.5	0.5	1.0	0.5

cent). Only 28.0 percent of the streptococci in the 12 cow feces were of the enterococcus group. *S. bovis* represented 41.1 percent of the total streptococcal density in the prison sewage during days when the laundry was operating. On other days, the *S. bovis* content was reduced to 4.0 percent. In domestic sewage from a suburban area of Cincinnati, Ohio, this organism represented only 0.5 percent of the fecal streptococci, whereas the enterococcus group, accounted for 97.5 percent.

When the values obtained for fecal coliform and fecal streptococci are projected into a ratio determination, several interesting relationships evolve. To compare such ratios for the prison sewage with those for domestic sewage, a series of four residential sewages were examined at seven daily periods. All data were reduced to median values and are reported in Table 34. The fecal coliform to fecal streptococci ratio for sewage from the prison dairy farm was 0.4, whereas that from the various residential areas varied from 4.3 to 8.6. This significant difference resulted from the variations in bacteria from animals living in various environments, which contributed the fecal coliform and fecal streptococci to the farm sewage samples. This dissimilarity between animals can be observed in Table 35, which gives estimated per capita contributions of indicator microorganisms in the feces of man and some farm animals. These data were taken, in part, from Geldreich et al. *(26)* and Kenner et al. *(91)* and were based on examinations of the same specimens. Note that the ratio of fecal coliform to fecal streptococci for man is 4.4, whereas that for other warm-blooded animals is 0.6 or less. These results indicate that farm wastes contain a large amount of pollution from animal sources, which support the findings for the prison dairy farm sewage.

TABLE 34. BACTERIAL DENSITIES IN VARIOUS SEWAGES

Sewage	Bacterial densities, count/100 ml			
	Total coliform	Fecal coliform	Fecal streptococci	Ratio FC/FS
Residential "A"	17,200,000	17,200,000	4,000,000	4.3
Residential "B"	33,000,000	10,900,000	2,470,000	4.4
Residential "C"	1,940,000	340,000	64,000	5.3
Residential "D"	6,300,000	1,720,000	200,000	8.6
Prison dairy farm	3,730,000	1,420,000	3,420,000	0.4

Discussion

The bacterial densities in the waste stabilization pond used in this study probably differ from those reported for other ponds because of the dairy operation. Because of this operation, the raw sewage contained a mixture of domestic and laundry wastes some of which were relatively highly chlorinated as a result of the bleaching process.

TABLE 35. ESTIMATED PER CAPITA CONTRIBUTION OF INDICATOR MICROORGANISMS FROM SOME ANIMALS[a]

		Average indicator density per gram of feces		Average contribution per capita per 24 hr		
Animals	Avg wt of feces/24 hr, wet wt, g	Fecal coliform, million	Fecal streptococci, million	Fecal coliform, million	Fecal streptococci, million	Ratio FC/FS
Man	150	13.0	3.0	2,000	450	4.4
Duck	336	33.0	54.0	11,000	18,000	0.6
Sheep	1,130	16.0	38.0	18,000	43,000	0.4
Chicken	182	1.3	3.4	240	620	0.4
Cow	23,600	0.23	1.3	5,400	31,000	0.2
Turkey	448	0.29	2.8	130	1,300	0.1
Pig	2,700	3.3	84.0	8,900	230,000	0.4

[a] Coliform data from Geldreich et al. (Ref. 26);
Fecal streptococci data from Kenner et al. (Ref. 91).

The basic purpose of our study, however, was to demonstrate a plan of bacteriological study for waste stabilization ponds. The results indicate a need for frequent sampling of the raw sewage intake of waste stabilization ponds. Such sampling would reveal the extreme fluctuations in microorganism densities during a 24-hour period and would allow calculation of more accurate geometric mean or median values. Past practice of the once-a-day grab sampling of raw sewage is an uncertain procedure that may lead to inaccurate interpretations of the data, and thus should be avoided.

Since soil abounds with many anaerobic and aerobic spore-forming bacteria that may ferment lactose in symbiosis with other organisms, some of the confirmed coliform test results may have included false positive reactions. In another survey of 251 soil samples (27), large discrepancies were frequently noted when results of the confirmed and completed MPN analyses of the same samples were compared. In our study, the raw sewage contained both domestic sanitary wastes and laundry wastes from washing work clothes that were exposed to both manure and field soils. Because of the possibility of false positive reactions from organisms associated with soil, the completed test is recommended for use in future studies of waste stabilization ponds. This test would yield greater accuracy in the estimation of the total coliform density and in the calculation of reduction efficiency. Data reported here also suggest that the use of the fecal coliform test and the fecal streptococcus procedure may yield more valuable information and may be more specific for warm-blooded-animal pollution appraisal than the use of a total coliform procedure alone.

Such ratios must be applied carefully since the bacterial densities in pollution wastes can be adversely affected when the pH is below 4.0 or above 9.0. Under these conditions, bacterial populations can be drastically reduced or altered by selective adaptation to the water environment by only a few strains. With these ground rules, certain ratio values can be applied to specific sources of warm-blooded-animal pollution. Ratios greater than 4:1 usually indicate the pollution is derived from domestic wastes, which are largely composed of man's body wastes, laundry wastes, and food refuse. Ratios of less than 0.6 suggest the pollution is derived from livestock and poultry wastes found on farm-feed lots and stockyards or from storm-water runoff. Further separation between stockyard discharges and storm-water runoff can be made by studying the distribution of fecal streptococci strains present in a given water sample.

The use of *S. bovis* as a specific indicator of non-human animal pollution may be a useful bacteriological procedure for special studies. Absence of *S. bovis* in the feces of dairy workers and its presence in feces of cows show this streptococcal species is not easily adapted to the environment in the human gut. The bovine feces were traced by inspection from the cow barns to the laundry, where they were transported on the workers' clothing, and were demonstrated in the sewage containing laundry wastes.

Summation

The use of several bacterial pollution indicators in a bacteriological study of a waste stabilization pond is demonstrated. A ¼-acre (0.1-hectare) pond located on a state-prison dairy farm received raw sewage composed of a mixture of domestic and laundry wastes. The relative density of four bacterial indicators of pollution were determined for four seasonal periods of the year.

Raw sewage coliform densities per 100 milliliters were 3.2 million in the spring, 3.3 million during the summer and autumn study periods, 2.4 million during the winter. Fecal coliform levels were between 790,000 and 920,000 per 100 milliliters for the spring, summer, and winter periods; for some undetermined reason the level increased in the autumn to 2.65 million. Fecal streptococcal densities varied from 2.1 to 4.1 million.

Reduction in coliform bacterial densities in terms of MPN varied from a low of 85.9 percent in the winter to a high of 94.4 percent in the autumn. Fecal coliform reductions were greater than 87.9 percent, whereas reductions of fecal streptococci were 97.0 percent or more.

Frequent samplings of raw sewage during each 24-hour period is recommended to provide more accurate average pollution indicator densities.

The absence of *S. bovis* in the feces of dairy workers, and its presence in the feces of cows and in the prison sewage that contained laundry wastes from washing dairy workers' clothing give additional statistical evidence of the importance of *S. bovis* as a specific indicator of non-human animal pollution in special studies.

Use of the fecal coliform test and the fecal streptococcus procedure in future waste stabilization pond studies may yield more valuable information than use of a total coliform procedure alone.

STORM WATER

Storm water is the major intermittent source of bacterial pollution that enters our nation's waterways. Rain falling to the earth picks up a few contaminants that are possibly derived from dust, pollen grain, and insects; these, however, contain less than one coliform per 100 ml of rain water (Geldreich, et al., in preparation). Once rain water reaches the earth, contaminants are added from numerous sources in the urban and rural environments of man. At the start of a rainfall following a dry period of several weeks or more, the bacterial-pollution levels are high. Then as the rain progresses, the density of pollution decreases to relatively low levels as a result of dilution.

In a study of storm water from a 27-acre residential and suburban business area free of sanitary and industrial waste discharges, fecal coliform and fecal streptococci averaged 11,000 and 21,000 per 100 ml, respectively(*144*). When storm-water discharge from this small area is projected to that representing the combined sewage — storm-water systems of large metropolitan areas, the pollution is intensified many-fold. In a special study(*145*) of a combined sewer system that serves approximately 25 percent of Detroit, Michigan, storm-water pollution levels over an 8-month period averaged 2,800,000 fecal coliform and 502,000 fecal streptococci per 100 ml.

Analysis of storm-water data for urban, residential, and rural areas (Geldreich, et al., in preparation) indicates the ratio of fecal coliform to fecal streptococci was less than 0.7 in separate storm-sewer systems and generally greater than 4 to 1 in combined-sewer systems. Since the fecal streptococcal densities were consistently higher than fecal coliform levels in separate storm-sewer systems, an analysis of 2,923 fecal streptococci isolated from such sources was performed. A total of 97.9 percent (2,861 strains) were *S. fecalis* and associated biotypes, which indicated warm-blooded animal pollution. The major source of fecal coliform bacteria in combined-sewer systems obviously was domestic wastes, while evidence indicates fecal contamination in separate systems was from dogs, cats, and rodents. In both the storm water and the fecal material from these animals, *S. fecalis* and associated biotypes showed the similar percentage patterns, and ratios of fecal coliforms to fecal streptococci were generally less than 0.7.

REVIEW*

Recent published reports(*146, 147*) on the fecal coliform bacteria have created renewed interest in this coliform group as a specific indicator of dangerous pollution. In 1948, personnel in our laboratory began a study of the elevated temperature procedure and soon observed that the EC medium was toxic to some of the attenuated fecal coliform strains. They observed that the necessary cell density for individual fecal coliform strains varied from 1 to as high as 100 before an inoculated EC tube showed elevated temperature growth with lactose fermentation. These observations led us to the use of prior enrichment in lactose or LST broth at 35°C to obtain optimum recovery of any level of fecal coliforms that might be in a water sample(*63, 70*). Similar observations on the necessity of prior enrichment were also reported by Levine and Minette(*65*) for the elevated temperature test using either EC or boric acid lactose broth.

Investigation was then directed toward the significance of the coliform strains other than + + − − type (*E. coli*). In one study(*63*) involving boric acid lactose broth (BALB), the reactions of 4,423 coliform bacteria from untreated surface water supplies at 14 treatment plants were recorded. In a similar study, the performance of EC medium at 45°C was evaluated on 5,794 cultures from these same raw-water supplies. Both media indicated a 93 percent detection of + + − − type (*E. coli*). Neither was, however, specific for *E. coli* since a few of each of the other IMViC types also gave a positive elevated temperature reaction. These results strongly suggest that the IMViC system of coliform classification did not adequately divide the coliforms found in waters into groups indicative of their source.

At this point, to study the distribution and occurrence in nature of these coliforms that would grow and produce gas at the elevated temperature became imperative. Data were thus collected on the reactions of 4,512 human, 2,339 livestock, and 1,896 poultry coliform strains isolated from 43 human, 32 livestock, and 28 poultry fecal samples(*26*). The EC procedure showed a 96.3 percent positive correlation and the BALB procedure, a 95.3 percent correlation with all types of coliforms from fecal sources. These findings suggest that the presence of EC- or BALB-positive coliform strains in water or wastes indicates relatively recent fecal pollution. Samples from 10 adult institutional residents who lived in a controlled environment, ate the same food, and worked on a dairy farm demonstrated the same coliform distribution as the other 33 samples from humans from varied environments. Human fecal samples contained the greatest variety of coliform types; in this case, 11 of the possible 16 type combinations. The feces of the other animals were almost exclusively + + − −, with only five other types occasionally appearing. Growth at elevated temperature did not appear to be specific for any one type or group of types and as a result, these procedures were detecting not only

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* This material was published previously in *Journal Water Pollution Control Federation* (Reference 153).

E. coli but other coliform types, which were also derived from warm-blooded-animal feces. The elevated temperature test was, therefore, a fecal coliform test and not an *E. coli*-type + + − − test.

When compared with the IMViC classification of coliform types, data on the elevated temperature test indicated this single test to be superior for fecal coliform detection. It did not require the isolation of a pure culture nor the multiple inoculation into different media for the four biochemical reactions. In fact, results of the elevated temperature test were accomplished in 24 hours as contrasted with the 5-day incubation period necessary for the methyl red reaction in the IMViC tests. These results suggested that the IMViC biochemical tests were not the best method to define fecal pollution in water and wastes. The elevated temperature test used as a confirmatory procedure was suggested as a possible solution.

Our current research program includes a study of the distribution and occurrence of fecal coliform bacteria in the feces of dogs, cats, and various rodents such as rabbits, chipmunks, and mice. This type of pollution could be a major source of the fecal coliform bacteria found in residential storm-water runoff and on bathing beaches. Preliminary data indicate the + + − − type (*E. coli*) to be the dominant coliform from pets and rodents. Only seven other coliform types have been found to date and few of these have failed to give a positive elevated temperature reaction. The data based on 948 strains examined indicate a 94.5 percent positive correlation of the test and the fecal origin of the coliform microorganisms.

Since soil may contribute large numbers of bacteria to drainage water, it is important to consider the correlation between coliform bacteria derived from nonpolluted soil and the fecal coliform test. Research data collected on 251 soil samples(27) from 26 states and 3 foreign countries indicated that fecal coliforms were usually absent or present in comparatively small numbers in unpolluted soils, with most results showing less than 2 fecal coliforms per gram. A marked increase was observed in the numbers of fecal coliforms in polluted soils sampled from various feed lots, locations recently flooded with domestic sewage, and river banks along heavily polluted streams. At these locations the density of fecal coliform varied from 3,300 to 49,000 per gram.

Intermediates have been reported to constitute frequently a considerable proportion of the coliforms in soils. In our study on soils, 48.1 percent of 2,348 strains from the unpolluted soils were the intermediate type − + − +. This type also comprised 51.6 percent of 289 coliform strains recovered from a protected raw-water supply for a large western city. The origin of the relatively high percentage of − + − + type coliform bacteria in this raw-water supply was probably the soil along the watershed. This conclusion is based upon finding 48.1 percent of this type in soils, the very low occurrence of − + − + type (0.6 percent) in fecal samples from warm-blooded animals, the comparitively low occurrence of fecal coliforms in the water source

(1.04 percent), and the occurrence of − + − + type in certain mountain streams studied at various times in the past. These data present, therefore, strong circumstantial evidence that the − + − + type of coliform is predominantly a soil type.

During periods of rainfall, pollution indicators that might be associated with vegetation could enter surface waters by way of storm-water drainage. In turn, many of the bacteria found on plants might result from insect contact. To study the occurrence of fecal coliforms from these sources, specimens of 152 species of plants and 40 insects were examined(*66*). These specimens were collected from various ecological environments and grouped into several categories. Results indicated that coliforms typical of the warm-blooded-animal gut contributed a relatively small percentage of the organisms associated with vegetation (14.1 percent) and insects (14.9 percent). A total of 1,203 coliform strains from vegetation and 1,084 coliform strains from insects were classified as to IMViC type and by the fecal coliform reaction. Although 13 different IMViC types were found on the vegetation samples and 14 different types on the insects, no type was predominant. These findings suggest that coliforms of the warm-blooded-animal gut, as measured by the fecal coliform test, contribute only a relatively small percentage of the organisms associated with vegetation and insects.

In fish, the occurrence of fecal coliform bacteria is apparently related to the habits and environments of the various species. Data collected thus far on fourteen species of fish from various locations in Canada, Tennessee, Ohio, and Pennsylvania, indicate that fecal coliform bacteria occur in greater numbers either in fish living in a polluted stream environment, or in fish that are "bottom feeders" or scavengers living in a relatively clean stream.

The elevated temperature test for fecal coliform bacterial densities may be performed by either the multiple-tube procedure or a recent development utilizing the membrane filter. The multiple-tube procedure consists of a confirmation of all positive lactose or lauryl tryptose broth tubes in EC medium incubated at 44.5°C for 24 hours. Production of gas is positive evidence of the presence of fecal coliforms. The use of boric acid lactose broth in a similar confirmatory procedure gives equivalent results; however, incubation temperature is reduced to 43°C and incubation time is extended to 48 hours with this medium. Because of the longer incubation time with no increased selectivity or sensitivity for BALB, we prefer the EC procedure. Fecal coliform densities are then determined by use of the most probable number tables.

Recently, we developed a fecal coliform procedure using the MF technique. This was developed in response to a desire by some laboratories to use the MF procedure for detection of total coliforms and to have available a similar MF procedure for the fecal coliform group. Until now, fecal coliform detection has been possible only by the multiple-tube (MPN) method.

This new fecal coliform procedure makes available another technique and thus gives water laboratory personnel a choice of methods for pollution studies. The fecal coliform tube confirmatory test may be expected to differentiate between coliforms of warm-blooded animals and coliforms from other sources with a 96 percent accuracy. The MF method has a 93 percent accuracy.

This MF procedure involves a new lactose medium (M-FC), which contains no inhibitory substances for coliform bacteria and depends on an incubation temperature of 44.5°C (± 0.5) for 24 hours (± 2) for its selectivity. The indicator system for lactose fermentation is aniline blue(*148*). Procedural details and a comparison with the multiple-tube method are described by Geldreich et al.(*149*).

From the information available and from our own investigations(*63, 70*) we have reached the following conclusions regarding the elevated temperature test:

1. The most acceptable temperature of incubation for separation of the fecal coliform group is 44.5°C in a water bath.
2. A small percentage of the fecal coliform strains will be excluded and an equal percentage of non-fecal coliforms will be included.
3. EC medium, described by Perry and Hajna, will give the most rapid results, as it requires only 24-hour incubation.
4. The test can be used only as a confirmatory procedure from coliform cultures growing on a nonselective medium.
5. In the evaluation of results, all coliforms from the feces of warm-blooded animals must be considered as fecal coliform strains, and all cultures isolated from unpolluted soils must be considered as non-fecal coliform strains.

Applying the above concepts as guides, Geldreich and associates (*26, 27, 66*) studied the coliform organisms isolated from the feces of several warm-blooded animals including humans, cows, sheep, pigs, chickens, ducks, and turkeys; from 223 soil samples with no known fecal pollution, collected in 26 States; from 28 fecally polluted soil samples from feed lots or locations recently flooded with domestic sewage; and from 152 species of plants and 40 species of insects. Coliform strains isolated from these samples were purified, and their reactions to *Standard Methods'* completed test, to IMViC, and to 44.5°C-temperature tests were determined. Results of these studies showed:

1. Coliform strains from feces gave good correlation of the elevated temperature test (fecal coliform) with the + + − − IMViC type and with the Parr fecal types (+ + − −, + − − −, and − + − −).
2. Coliforms from soils showed good correlation of the elevated temperature test with the IMViC type + + − − and with the Parr fecal types, but attempts to classify the coliform group by individual reactions were unsuccessful.

3. The most prevalent IMViC types found in unpolluted soils were −+−+ type (48.1 percent) and −−++ (18.8 percent).

4. Small numbers of coliforms were found on plants, but very few of these were classified as fecal coliforms.

5. The number of coliforms recovered from insects showed wide variation. Relatively few fecal coliforms were found.

6. No IMViC type was predominant on either vegetation or insects.

Separation of fecal coliforms from non-fecal coliforms apparently can be obtained with essentially equivalent results by using the IMViC types and interpreting the results according to the "Parr classification," or by using the elevated temperature test, which was superior in simplicity of technical procedure and in time required to complete it.

The mass of data now accumulated regarding the elevated temperature test, indicates that the separation of fecal from non-fecal coliform bacteria can be readily accomplished. The presence of fecal coliform organisms in untreated water should be considered as an indicator of recent fecal pollution. No method is currently available, however, that can be used to differentiate fecal coliforms of human origin from those of other warm-blooded animals. The elevated temperature test is, therefore, a measure of fecal coliforms from all kinds of warm-blooded animals, man included. Untreated water supplies that do not contain fecal coliforms, but do contain various densities of other coliform organisms may indicate contamination from relatively less recent fecal pollution or from organisms from the adjacent soil.

The presence of any type of coliform organism in treated water suggests either inadequate treatment or postchlorination contamination in the system(*69*). It is true there are some differences between various coliform strains with regard to natural survival and their chlorination resistance, but these are minor biological variations that are more clearly demonstrated in the laboratory than in the water-treatment system. The presence of any coliform bacteria, fecal or non-fecal, in treated water should not be tolerated.

The fecal coliform procedure probably has its greatest application to investigations of river pollution, sewage treatment systems, and bathing water quality. In these investigations, the bacteriological objectives are to measure the degree of potential hazard to public health. From the information now available, the elevated temperature test, in conjunction with the total coliform determination, yields the best specific data on the proportion of fecal pollution with which to discriminate between pollution derived from warm-blooded animals and that from the myriad of other pollution sources.

The significance of fecal coliform bacteria in pollution investigations is now firmly established by the legal hearings held at Sioux City, Iowa,(*71*) and at St. Joseph, Missouri,(*72*) in 1959. In future pollution studies, we will find an increasing use for these newer research methods and indeed will go beyond the scope of the objectives and procedures in *Standard Methods* in attempts to detect better the probable source, degree, and potential hazard of various pollutants to public health.

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SUBJECT INDEX

- Acetylmethylcarbinol, *see* Voges Proskauer Test
- Aftergrowth of bacteria, *see* Multiplication of bacteria
- Apparatus, 25, 34
- Application of pollution indicators
  - polluted water, 43, 45, 109
  - treated water, 42, 45
- Bacterial contributions, per capita
  - livestock, 51, 53
  - man, 51, 53
  - poultry, 51, 53
- Bacterial density, *see* Pollution indicator density
- Bacteriological media
  - use with membrane filter procedure, 21, 33, 34
  - use with multiple tube procedure. 19, 23, 30
  - streptococci, 58
- Bathing water, 106
- Biochemical test, 3
- Citrate test, 10
- Coliform density, *see* Multiple-tube procedure
- Coliform IMViC types
  - classification of,
    - drinking water 93, 94
    - fish, 64, 66, 67-69
    - insects, 77-79
    - livestock, 48-51
    - man, 48
    - pets and rodents, 104
    - poultry, 48, 59, 51
    - soil, 87, 90, 91, 106
    - sewage, 101
    - storm water, 106
    - vegetation, 75, 77-79, 107
    - waste-stabilization ponds, 98-100
  - definitions, 12-14, 17, 20, 21
  - detection, *see* tests for
  - index of fecal pollution, 1, 42, 108
  - occurrence in
    - drinking water, 93, 94
    - fish, 64, 66, 67
    - insects, 75, 77, 78, 79
    - livestock, 48-51, 53
    - man, 48
      - population change, 48, 51
    - pets and rodents, 104
    - poultry, 48, 49, 51
    - soil, 87, 90, 91
    - storm water, 106
    - vegetation, 75, 77-79
  - persistence in, 61-63
  - significance of, 16, 17, 25, 26, 29-33, 48-51, 64, 66, 67, 75, 77, 78, 79, 87, 90, 91, 93, 94, 105, 106, 108, 109
  - tests for, 18-22
    - multiple-tube procedure, 20
    - membrane filter procedure, 20
- Coliform tests, 3
  - membrane filter, 21
  - multiple tube, 20
- Completed test, *see* Multiple-tube procedure
- Confirmed test, *see* Multiple-tube procedure
- Definition of terms, 18
  - coliform bacteria, 20
  - completed test, 21, 43
  - confirmed test, 20, 43
  - elevated temperature test, 21
  - fecal coliform bacteria, 22, 95
  - fecal streptococci, 69, 95
  - IMViC, 13
  - membrane filter procedure, 21
  - presumptive test, 20, 43
- Differentiation of coliforms, by
  - biochemical test, 3
  - citrate test, 10
  - coliform test, 3
    - membrane filter, 21
    - multiple tube, 20
  - elevated temperature test, 4, 14-17, 44, 108
  - gas ratio test, 4
  - IMViC classification, 12
  - indole reaction, 6-8
  - methyl red test, 5
  - purification of cultures, 24, 31, 47. 58, 75, 86
  - Voges-Proskauer test, 8-10
- Differentiation of streptococci by
  - growth at 10°C, 67-69, 79-82, 96, 97
  - growth at 45°C, 67-69, 79-82, 96, 97
  - growth at 6.5% NaCl, 67-69, 79-82, 96, 97
  - peptonization, 67-69, 96, 97
  - purification of cultures, 58, 75, 96
  - starch hydrolysis, 67-69, 83, 96, 97
- Domestic sewage, 100, 101
- Drinking water, 42, 45, 93, 94, 109

Elevated temperature test, 4, 14-17, 44, 108
Enterococci, *see* Fecal streptococci; Fecal streptococci strains
Environment
  soil, 85
  water, 93
Equipment, 25, 34
Evaluation of fecal-coliform methods, 25, 26, 31, 39, 41-45, 91, 105, 106, 108
Farm animals, 48-51, 53, 101; *see also* Bacterial contributions, per capita; Coliform IMViC types; Fecal coliforms; Warm-blooded animals.
Fecal coliforms
  choice of procedure, 23, 107
  classification of
    drinking water, 93, 94
    fish, 64, 66, 67
    insects, 75, 77, 78, 79
    livestock, 48-51, 53
    man, 48
    pets and rodents, 106
    poultry, 48, 49, 51
    sewage, 101
    soil, 87, 90, 91
    storm water, 104, 106
    vegetation, 75, 77, 78, 79
    waste stabilization ponds, 98-100
  definitions, 17, 20, 21
  differentiation, *see* Differentiation of coliforms
  detection, *see* tests for
  index of fecal pollution, 1, 42
  occurrence in
    cats, 104, 106
    dogs, 104
    drinking water, 93, 94
    fish, 64, 67, 69-72, 107
    insects, 75, 107
    livestock, 48-51, 53
    man, 51, 53
    poultry, 101
    rodents, 104, 106
    sewage, 101
    soil, 87, 106
    vegetation, 75, 107
  persistence in, 61-63
    bluegill, 61, 63
    carp, 61, 62
  significance of, 79, 89, 94, 105, 108
  tests for, 18, 22
    membrane-filter procedure, 21, 23
    multiple-tube procedure, 20
Fecal coliform-fecal streptococci index, *see* Ratios
Fecal streptococci
  classification of
    fish, 67-69
    insects, 69, 79-82
    sewage, 100
    storm water, 104
    vegetation, 79, 81
  definitions, 69, 95
  detection, *see* tests for
  differentiation, *see* Differentiation of streptococci
  occurrence in
    fish, 59
    insects, 75
    sewage, 101
    vegetation, 75
    waste-stabilization ponds, 98-100
  persistence in
    bluegill, 61, 63
    carp, 61, 62
  significance of, 69, 83, 100, 101, 103
    peptonization, 69, 83
    starch hydrolysis, 83
  tests for
    azide dextrose broth, 58, 74, 96, 97
    ethyl violet azide medium, 58, 74, 96, 97
    KF agar, 58, 74, 96, 97
Fecal streptococci strains
  Enterococci, 69, 96, 100
  S. anginosus, 100
  S. bovis, 69, 96, 100, 101, 103
  S. equinus, 69, 100
  S. fecalis, 58, 69, 83, 104
  S. lactis, 69, 83
  S. mitis salivarius, 69, 100
Fish, *see* Fresh-water fish
Fresh-water fish, 57
  classification of streptococci, 67-69
  contributions of polluted fish to potable water, 69-72
  densities per gram
    coliforms, 59
    fecal coliforms, 59
    fecal streptococci, 59
  density variations in upper and lower gut, 61, 64, 65

- occurrence in coliform types in fish, 64, 66
  - and their water environment, 64, 67, 69-72
- percentile distribution of IMViC types, 64-67
- persistence of indicators in fish, 59
  - enteric pathogens, 59, 61-63
  - fecal coliforms, 59, 61-63
  - fecal streptococci, 59, 61-63
- pollution indicator density, 59, 61, 64, 65, 69-72

Gas ratio test, 14
Ground water, 36, 70-72
Hydrogen: carbon dioxide, *see* Gas ratio test
IMViC classification
- comparison with fecal coliform test, 12, 16, 25, 51, 108, 109
- comparison with multiple-tube procedure
  - BALB, 25, 47-52, 54, 77, 105
  - E.C., 32, 47-52, 54, 77, 107
- for coliform bacteria, 13
- IMViC definition, 12
- series of tests
  - citrate test, 12
  - indole reaction, 12
  - methyl red test, 12
  - Voges-Proskauer test, 12
- validity of IMViC classification, 14, 52, 54, 77, 106

Incubation temperatures, 25, 31, 33, 34, 41, 43, 97
Indicator density, *see* Pollution indicator density
Indole reaction, 6
Insects, 73
- classification of streptococci, 79-82
- density per gram
  - coliforms, 75
  - fecal coliforms, 75
- percentile distribution of IMViC types, 77

Intermediate coliform strains
- classification of intermediate *Aerogenes-cloacae* (IAC), 93
- occurrence, *see* Coliform IMViC types

Interpretation of fecal coliforms, of fecal streptococci, of total coliforms, *see* Sanitary significance of
Lagoon, *see* Waste-stabilization ponds
Legal status
- fecal-coliform procedure, 44, 109

Media preparation, *see* Bacteriological media
Membrane-filter procedure, 21, 33
- accuracy, 39, 108
- detection of
  - coliforms, 21
  - fecal coliforms, 33
- false positive reactions, 38
- media, 21, 33, 34
- m-FC medium, 34, 107
- MPN vs. MF, 20, 35-37
- sample volume limitation, 39-41

Methyl red test, 5
MPN, *see* Multiple-tube procedure
Multiple-tube procedure, 20, 21, 43
- accuracy, 25, 26, 31, 39, 44, 91, 105, 106
- BALB confirmatory procedure, 23, 25, 54
- EC confirmatory procedure, 30, 32, 54
- EC vs. BALB, 33, 47-52, 54, 105, 107
- false positive reactions, 83, 102
- media, 19, 23, 30
- MPN vs. MF, 20, 35-37, 39
- prior enrichment, 54, 105

Multiplication of bacteria, 59, 61-64
- antagonistic effects, 61
- time-temperature effects, 69

Overcrowding of membrane filter colonies, 39-41
Oxidation ponds, *see* Waste-stabilization ponds
Pathogenic bacteria, 1, 61-63, 70, 94
Physiology of bacteria, *see* Differentiation of coliforms; Differentiation of streptococci
Pollution indicator application, *see* Application of pollution indicators
Pollution indicator density
- drinking water, 93
- fish intestinal material, 59, 61, 64, 65, 69-72
- ground water, 36
- insects, 75
- livestock, 51, 53, 101
- man, 51, 53, 101
- poultry, 51, 53, 101
- sewage, 101
- soil, 87-89

  storm water, 104
  vegetation, 75
  waste-stabilization pond, 98, 101
Purification of cultures, *see* Differentiation of coliforms; Differentiation of streptococci
Ratios
  sewage, 101-103
  storm-water runoff, 104
  warm-blooded animals
    chicken, 101-103
    cow, 101-103
    duck, 101-103
    man, 101-103
    pig, 101-103
    sheep, 101-103
    turkey, 101-103
Salmonella organisms, *see* Fresh-water fish, persistence of indicators
Sampling of sewage, 99, 100, 103
Sanitary significance of
  fecal coliforms, 51-54, 79, 89, 94, 105, 108
  fecal streptococci, 69, 83, 100-103
  total coliforms, 3, 51, 64-67, 83, 91, 102
Seasonal variations of pollution indicators
  sewage, 98
  soil, 87
Sewage, *see* Waste-stabilization ponds
Sewage effluents, 98-100
Shigella organisms, *see* Fresh-water fish, persistence of indicators
Soils, 85
  bacterial indicator densities per gram, 87-89
  coliform IMViC type, 87
  sampling procedure, 86
  sources, 86
  time relationship to bacterial density, 98
Stabilization ponds, *see* Waste-stabilization ponds
Storm water, 104, 106
  fecal coliforms, 104
    pets and rodents, 100, 104
  fecal streptococci, 104
    pets and rodents, 106
  total coliforms, 104
Stream Surveys, 19, 21
Streptococci, *see* Fecal streptococci
Surface water, 1, 24, 28, 31, 35-37, 45
Temperature incubation, *see* Incubation temperatures
Total coliforms, *see* Coliform IMViC types
Vegetation, 73
  classification of streptococci, 79, 81
  density per gram
    coliforms, 75
    fecal coliforms, 75
  percentile distribution of IMViC types, 75
Verification of colonies, 35, 36-39
Voges-Proskauer test, 8
Warm-blooded animals, 47-54; *see also* Bacterial contributions, per capita; Coliform IMViC types; Fecal coliforms;
  classification of fecal streptococci, in feces of
    cow, 100
    man, 100
    pets and rodents, 104
  coliform daily per capita discharge, 51, 53
  coliform population changes in human feces, 48, 50
Waste-stabilization ponds, 95
  bacterial reduction in pond effluent, 98-100
  classification of fecal streptococci, 100
  frequency of sampling, 99, 100, 103
  pollution indicator loadings, 98
  seasonal bacterial discharge rates, 98
  sewage, 24, 36, 50, 101, 104
Water, *see also* Bathing water; Ground water; Surface water
  analysis, 20, 21, 58

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